VOLUME 37 MAY 1959 NUMBER 5

Canadian Journal of Biochemistry and Physiology

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

Canadian Journal of Biochemistry and Physiology

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Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 37 MAY 1959 NUMBER 5

A COMPARISON OF THE UTILIZATION OF RAPESEED OIL AND CORN OIL BY THE RAT[1]

JOYCE L. BEARE, T. K. MURRAY, H. C. GRICE, AND J. A. CAMPBELL

Abstract

The effects of Golden rapeseed oil and corn oil on weekly weight gains, food consumptions, liver storage of vitamin A, plasma and adrenal cholesterol concentrations, organ weights, and testes histology were determined in Wistar rats for 5 weeks after weaning. At each weekly interval, animals fed rapeseed oil showed lesser weight gains and food consumptions, but, when body weight gains were adjusted for food consumptions by covariance analyses, differences largely disappeared. Liver storage of vitamin A was similar with both oils. Testes of animals fed rapeseed oil exhibited no alteration in cellular characteristics, but a reduction in tubular size. When fed to older rats, rapeseed oil again produced smaller weight gains which were accounted for at 4 weeks' time by a depressed appetite.

Similar weight gains were obtained with corn oil and rapeseed oil when the oils were paired fed and when adjustments for food consumption were made by covariance analyses of weight gains of animals receiving the oils ad libitum. With corn oil supplied on a restricted basis the testicular tubules were smaller than those obtained with unrestricted feeding of corn oil. The absorption of corn oil and rapeseed oil fed ad libitum was 95 and 92% respectively. It was concluded that the two oils were not very differently utilized.

Introduction

The continued interest in rapeseed oil as a food prompted further investigations of its nutritional properties. Previous work in this laboratory (1) showed that in the albino rat, rapeseed oil at levels of 0.5 to 10% by weight of a purified diet had negligible effects on body weight gain, food consumption, and lipid excretion. At the higher levels of 16 and 20% oil, weight gains and food consumptions were significantly lower for rapeseed oil than for corn oil. There were no differences in adrenal weights after the animals had been on diet for approximately 10 weeks, an unexpected finding in view of the observations of Carroll that rats fed rapeseed oil for 4 weeks had larger adrenals (2). It was therefore decided to study rats fed rapeseed oil and corn oil at weekly intervals after weaning to determine the time when differences appeared. At the same time the liver storage of vitamin A administered with each oil was determined. To ascertain whether the age of the animals receiving rapeseed oil influenced the response to it, the oil was also fed to older rats.

[1]Manuscript received November 7, 1958.

Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa. This article is based on a paper presented at the 41st Annual Conference and Exhibition of The Chemical Institute of Canada, Toronto, May 26–28, 1958.

In order to assess the significance of the reduced food consumptions of animals fed rapeseed oil, two procedures were investigated, that of paired feeding and that of weight-gain adjustment by covariance analyses for differences in food consumptions of animals fed ad libitum.

Since there is a lack of agreement in the coefficients of digestibility reported by others for rapeseed oil and for the methyl ester of its predominant fatty acid, erucic acid (3, 4, 5, 6), it seemed important to investigate further the lipid excretion with each dietary oil.

It is the purpose of this paper to describe experiments involving a total of 220 rats in which the relative utilization of rapeseed oil and corn oil was compared.

Procedures and Results

Inbred rats of the Wistar strain, housed in individual screen-bottomed cages, were randomized in blocks, each of which consisted of animals of the same initial body weight and litter so far as possible. Block differences were removed in the statistical analyses. Canadian-grown rapeseed oil of the Golden strain[2] or corn oil was incorporated into the purified basal diet, which consisted of the following in % by weight: oil, 20; cornstarch, 30; sucrose, 20; casein, 19; vitamin mixture in casein,[3] 1; salt mixture, U.S.P. XIV, 4; alphacel,[4] 6.

With the exception of the paired-fed animals of experiment III, the animals were fed ad libitum. In experiment I vitamin A in doses of approximately 200 units in 0.1 ml of rapeseed oil or corn oil was administered by mouth twice weekly, and the amount in the liver determined (7). Otherwise all vitamins, including the fat-soluble ones, were mixed with casein for incorporation into the diet.

The neutral lipid content of weekly fecal collections from individual rats was determined by a 5-hour petroleum ether extraction of the dried, powdered material in a Goldfisch apparatus. Further extraction with 5% acetic acid in petroleum ether, as described by Hopkins, Murray, and Campbell (8), gave an estimation of fatty acids from soaps.

At the termination of each experimental feeding period, the animals were sacrificed by ether anaesthesia, and blood was drained from a neck incision. Livers, adrenals, and testes were removed and weighed. Concentrations of plasma and adrenal cholesterol were determined by ferric chloride methods (9, 10). Testes were examined histologically, photomicrographs were made, and tubules counted per microscopic field.

Experiment I. Weekly Changes in Young Rats

One hundred male weanling rats, 21 to 24 days of age, were paired according to initial body weight, one of each pair supplied with the diet containing corn

[2]A selection of the Argentine variety supplied through the courtesy of Dr. B. M. Craig and the Saskatchewan Wheat Pool.

[3]100 g vitamin mixture contained: 100 mg thiamine, 100 mg riboflavin, 100 mg pyridoxine HCl, 300 mg calcium pantothenate, 5 g inositol, 500 mg niacin, 1 g *p*-aminobenzoic acid, 2 mg biotin, 0.2 mg vitamin B_{12}, 10 g choline chloride, 50,000 I.U. vitamin A, 10,000 I.U. vitamin D, 1000 I.U. vitamin E.

[4]Non-nutritive cellulose obtained from Nutritional Biochemicals Inc.

oil and the other with the diet containing rapeseed oil. As shown in Table I, the animals fed rapeseed oil exhibited lower accumulated weight gains and food consumptions after each week than those fed corn oil. Weight gains from the first to second and from the second to third weeks were greater for the corn oil fed rats, but were similar for both groups of rats for the fourth and fifth weeks. The increasing similarity in the weekly weight gain increments and the greater intake of food seem to indicate an adaptation to rapeseed oil.

TABLE I

Mean weight gains, food consumptions, and organ weights of rats fed corn oil (CO) and rapeseed oil (RSO)

Weeks on diet	Dietary fat	Food consumption, g	Weight gain, g		Liver wt., g		Adrenal wt., mg/pair	Testes wt., g/pair
			Unadj.†	Adj.‡	Unadj.	Adj.		
1	RSO	28.7**	14.1**	15.4	2.17	2.22**	14.8	0.38**
	CO	31.9	17.5	16.2	2.04	1.97	14.9	0.46
2	RSO	71.3**	32.1**	35.9*	2.59	2.65	15.0	0.67**
	CO	89.2	46.6	42.8	2.75	2.69	15.3	0.90
3	RSO	120.0**	47.5**	58.8	3.19**	3.69**	19.1	1.15**
	CO	160.4	76.6	65.3	3.68	3.18	20.7	1.44
4	RSO	174.0**	65.3**	80.9	3.72**	4.57**	18.1**	1.39**
	CO	230.8	100.5	84.9	4.43	3.57	23.2	1.80
5	RSO	231.5**	86.5**	96.1	4.43**	5.29**	25.1	1.75**
	CO	282.5	121.7	112.1	4.69	4.25	24.8	2.27

* and ** indicate that the mean for RSO is significantly different from that for CO at $P=0.05$ and $P=0.01$ respectively.
†Unadj.: the mean of the absolute weights.
‡Adj.: the mean adjusted for food consumption by covariance analyses.

When weight gains were adjusted for food consumption by covariance analyses, it was shown that the amount of the food intake entirely accounted for the weight gains except at the second week. Absolute liver weights were significantly greater for the corn oil fed animals at 3, 4, and 5 weeks' time. Statistical adjustment of both groups to the same body weight indicated that the rapeseed oil group had significantly heavier livers. During the first 5 weeks after being weaned the rats receiving rapeseed oil did not have larger adrenals and at the fourth week had significantly smaller ones, the difference being eliminated by covariance analysis. The testes were significantly smaller for the rapeseed oil rats at each week.

As shown in Table II, the liver storage of orally administered vitamin A was similar with both oils. At 3 weeks' time and thereafter, the cholesterol concentration in the adrenals was greater in rapeseed oil fed animals. Plasma cholesterol values were similar for the two oils. In animals receiving rapeseed oil there was also an increased excretion of neutral lipid by the third week but no significant changes in the soap fraction at any time.

Histological studies indicated that the testes of the rapeseed oil fed rats retained the normal cellular morphology. No differences were observed in the shape, size, and staining properties of Sertoli, Leydig, and spermatogenic cells. As shown in Fig. 1, tubular size was less with rapeseed oil than with corn oil. The average tubule counts for rats fed corn oil and rapeseed oil at

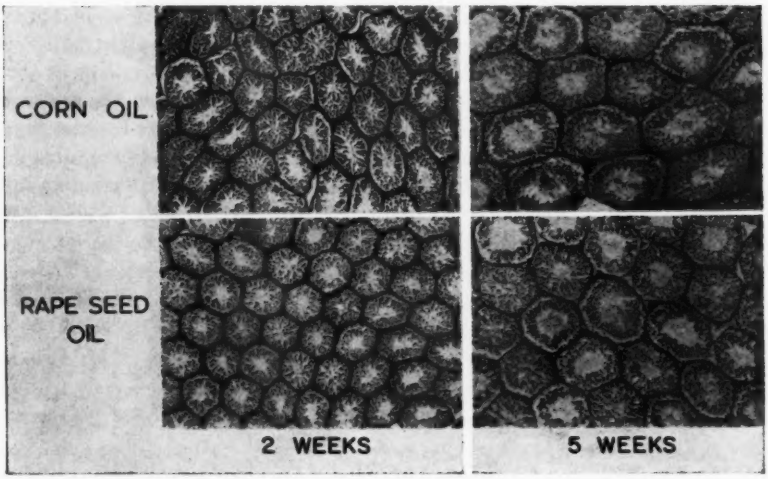

FIG. 1. Testes of rats fed 20% corn oil and rapeseed oil for 2 and 5 weeks (haematoxylin–eosin stain).

TABLE II

Mean levels of liver vitamin A, adrenal cholesterol, and fecal neutral lipid of rats fed corn oil and rapeseed oil

Weeks on diet	Dietary oil	Liver vitamin A, I.U./liver	Adrenal cholesterol, mg/100 g tissue	Fecal neutral lipid, g/rat/week
1	RSO	314	1.98	.43
	CO	291	1.14	.20
2	RSO	101	2.27	.45
	CO	121	1.20	.35
3	RSO	147	4.07**	.59**
	CO	137	1.45	.29
4	RSO	149	5.03**	.66**
	CO	150	1.68	.28
5	RSO	—	4.02**	.74**
	CO	—	1.52	.31

**Means for RSO significantly different at $P = 0.01$.

2 weeks were 43.1 and 50.8 respectively, at 3 weeks 41.3 and 49.0, at 4 weeks 35.2 and 39.0, and at 5 weeks 20.4 and 24.4. These differences were statistically significant.

Experiment II. Effect on Older Rats

To test the effect of rapeseed oil on more mature rats, 30 males ranging from 120 to 163 g were paired according to weight and litter, one of each pair being supplied with the corn oil diet.

Data on this test are given in Table III. Statistical analyses of weight gains and food consumptions during the first 4 days showed that the rapeseed oil fed animals ate less and gained less, and that the standard error of the

means of the weight gains was less than one gram. The weight gains were still significantly different when corrected for food consumption. At 4 weeks the mean weight gain for corn oil was significantly greater than that for rapeseed oil, but was not significantly different when adjusted for food consumption. This is perhaps evidence of adaptation as seen in the study of younger rats.

TABLE III

Food consumptions, weight gains, testes weights, adrenal weights, and adrenal cholesterol concentrations of paired, adolescent rats fed 20% corn oil or rapeseed oil

Dietary oil	Food consumption, g, in:		Weight gain, g, in:				Testes wt., g/pair	Adrenal wt., mg/pair	Adrenal cholesterol, mg/100 g tissue
			4 days		4 weeks				
	4 days	4 weeks	Unadj.†	Adj.‡	Unadj.	Adj.			
RSO	43**	298**	20**	20**	75**	81	2.59**	36.4	3.97**
CO	54	335	23	23	104	99	2.84	36.0	2 00

†Unadj.: the mean of the absolute weights.
‡Adj.: the mean adjusted for food consumption by covariance analyses.
**Means significantly different at $P = 0.01$.

Animals fed rapeseed oil had smaller testes, but adrenals similar in size to those fed corn oil. Since the regression coefficients of organ weights on body weights were extremely small, the mean organ weights were not adjusted. Rapeseed oil produced a significantly greater adrenal cholesterol concentration, but no change in plasma cholesterol.

Experiment III. Paired Feeding with Rapeseed Oil and Corn Oil

To compare weight gains obtained by paired feeding with those adjusted by covariance analyses for food consumption of animals fed ad libitum, a study was carried out with weanling male rats, 21 to 24 days of age, arranged in 15 randomized triplicates. Each comprised a rat fed the corn oil diet ad libitum, a rat fed rapeseed oil ad libitum, and a rat whose intake of the corn oil diet was restricted to the amount the animal receiving rapeseed oil consumed on the previous day. A second study of the same plan was carried out, and the results of both shown in Fig. 2 as studies A and B.

It was observed that the two ad libitum fed groups behaved as in other tests, that is, the rats receiving the 20% corn oil diet gained significantly more than those receiving the 20% rapeseed oil diet. The corn oil diet, when restricted in amount to that of the rapeseed oil diet, appeared to be little better than the latter. Analyses of variance indicated no difference in weight gain response. The unadjusted means of weight gains for the groups, corn oil ad libitum, rapeseed oil ad libitum, and corn oil paired with rapeseed oil ad libitum, and the means of the two ad libitum groups adjusted to equal food consumption by covariance analyses are shown separately. From the two studies it is seen that after the first week, paired feeding and adjustment of gains of ad libitum fed animals to the same food consumption by covariance analyses yielded similar results. They showed no real difference between the oils with respect to weight gains relative to food consumptions.

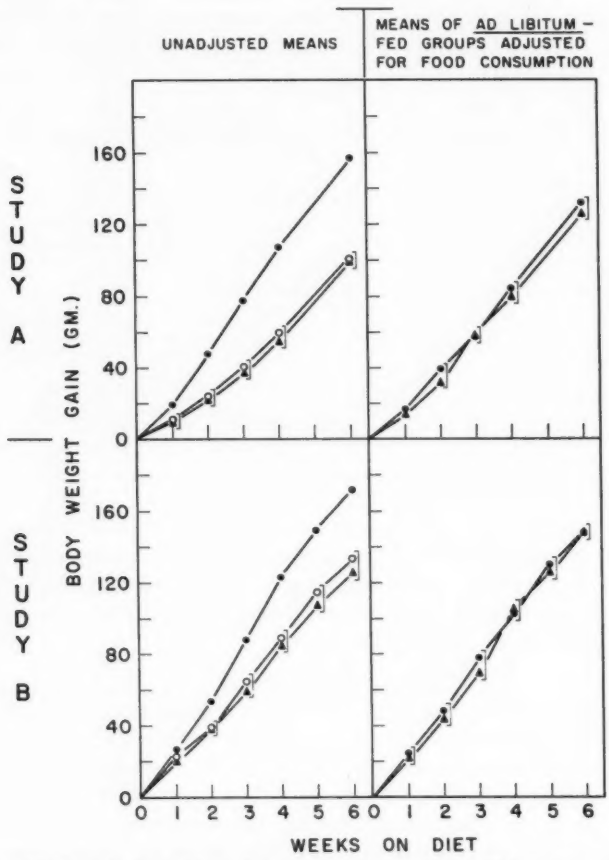

FIG. 2. Absolute body weight gains of rats fed corn oil ad libitum, rapeseed oil ad libitum, and corn oil paired with rapeseed oil, and weight gains of ad libitum fed groups adjusted for food consumptions. Brackets indicate means which are statistically not different at $P=0.01$.

Mean absolute organ weights are shown in Table IV. In each study the consistent significant changes in absolute organ weights were: the livers of animals fed corn oil ad libitum were larger than those of the other two groups; the testes of rapeseed oil fed rats were smaller than those of rats on the restricted corn oil diet, which were in turn smaller than those fed corn oil ad libitum; spleen weights were greatest in the rapeseed oil fed animals. Organ weights were not adjusted for body weights because, after 6 weeks of experimental feeding, the regression coefficients were found to be not significantly different from zero.

The mean tubule count for the group fed corn oil ad libitum was 19.3, for the group fed rapeseed oil ad libitum 20.7, and for the group fed restricted

amounts of corn oil 22.2. With the Duncan test (11) it was shown that a significant difference existed only between the two corn oil groups.

TABLE IV

Mean organ weights of animals fed corn oil ad libitum, rapeseed oil ad libitum, or corn oil paired fed with rapeseed oil

Dietary oil	Study	Liver wt., g	Testes wt., g/pair	Spleen wt., g	Adrenal wt., mg/pair
CO ad lib.	A	10.23	2.31	—	32.2
	B	13.10	2.56	0.44	32.3
RSO ad lib.	A	8.14	1.69	—	27.9
	B	10.80	2.24	0.56	29.0
CO restricted	A	6.44	1.85	—	23.8
	B	10.07	2.43	0.51	27.2

Determinations of the lipid excretion for rats in the last study, shown in Table V, demonstrated that all coefficients of digestibility were greater than 90%, though that for rapeseed oil was significantly lower than that of the other two groups. This was accounted for by an increased amount of neutral lipid material excreted. The restricted animals excreted the least lipid material, but the absolute amount was not great enough to alter significantly the coefficient of digestibility from that of the rats fed corn oil ad libitum.

TABLE V

Mean lipid excretion of rats fed corn oil ad libitum rapeseed oil ad libitum, and corn oil restricted

Dietary oil	Oil consumed, g/rat/wk	Lipid excreted, g/rat/wk			Apparent digestibility, %
		Neutral lipid	Soap	Total	
CO ad lib.	18.3	0.539	0.318	0.857	95.1
RSO ad lib.	14.9	0.844**	0.240	1.084**	92.6**
CO restricted	14.5	0.349	0.203	0.552	95.9

**Mean for RSO significantly different from that for CO at $P = 0.01$.

Discussion and Conclusions

In each experiment it is seen that the weight gain of rats supplied ad libitum with 20% rapeseed oil differed from that of rats supplied with 20% corn oil because of the relative amounts of the respective diets consumed. This finding is in agreement with previous work from this laboratory (1) and with that of Thomasson (12), who showed that the ratio of body weight gain to food calories was essentially the same for most oils, including rapeseed oil.

The chief effect of rapeseed oil was an apparent depressed appetite which occurred early in the feeding periods of both young and older rats. Wood and Migicovsky demonstrated an early effect of rapeseed oil on cholesterol metabolism in the rat (13). Although animals receiving rapeseed oil had a much slower rate of gain initially, there appeared to be an adaptation to the oil, as exhibited by the similar weekly weight gains with the two oils later in the experimental period. When the intake of the corn oil diet was restricted to

that of the rapeseed oil diet the weight-gain-promoting qualities of the two oils were essentially the same. After the initial stages of the studies, actual paired feeding and statistical adjustment of gains for differences in food consumption to the same amount gave similar results for both oils. The requirement for fewer rats and only two feeding periods per week, rather than individual daily paired feedings, is an advantage of the latter method. The food restriction imposed upon the paired-fed animals resulted in an intake that was approximately 80% of that consumed by animals receiving the corn oil diet ad libitum.

To determine the best way of expressing organ weights, a matter emphasized by Heroux and Gridgeman (14), adjustments for body weight were investigated by covariance analyses. When the regression lines were parallel and the coefficients of appreciable magnitude, the adjusting of organ weights was justified. Since both conditions were not attained for adrenals and testes, their unadjusted weights were used.

Contrary to the findings of Carroll with Sprague Dawley rats (2), it was shown that the adrenals of Wistar rats fed rapeseed oil were at no time significantly greater in weight than those of animals fed corn oil. The testes of rats fed rapeseed oil were smaller. The smaller tubules found in experiment I could be indicative of a decreased sperm production although current reproduction studies in our laboratory do not support any theory involving sterility of rapeseed oil fed animals. In experiment III, when the animals were 6 weeks past weaning the difference between the animals fed corn oil and rapeseed oil ad libitum was not significant, but the tubules of the restricted animals were significantly smaller than those of the animals on the same diet supplied ad libitum. It would, therefore, seem that food intake was the main factor responsible for differences in tubule size.

Since the liver storage of vitamin A was similar with both oils, there appeared to be no adverse effect of rapeseed oil on the absorption of vitamin A, and consequently no indirect effect of the vitamin on weight gain.

For corn oil and rapeseed oil fed ad libitum and for corn oil fed in the restricted manner, the apparent digestibilities of the oils were all over 90%. Such coefficients of digestibility are called apparent because no adjustment was made for the not very precise measurement of metabolic fat. This correction would have theoretically given rise to a still higher value for the digestibility of each dietary oil. Carroll, who fed 25% rapeseed oil in a basal fox cube diet and determined metabolic fat, found that the coefficient of digestibility of the oil was 58% (4). With diets similar in the content of fat, protein, and U.S.P. XIV salt mixture to those reported in these studies, Howard[5] estimated the minimal, apparent digestibility of rapeseed oil to be 88.3%. Murray *et al.* (6) found the coefficient of digestibility of methyl erucate to be 90% in Wistar rats, whereas Carroll found it to be 59% in Sprague Dawley rats. It was demonstrated by Carroll that the higher the calcium intake in a diet containing erucic acid the greater the excretion of fat (15). Since this fact does not

[5]Private communication from H. W. Howard, The Borden Company, N.Y.

explain differences between laboratories where diets of similar calcium content have been tested, the possible effects of strain differences assume greater importance.

The finding that equal quantities of rapeseed oil and corn oil produced similar weight gains, as shown by paired feeding and covariance analyses of ad libitum fed animals, was consistent with both corn oil and rapeseed oil having high coefficients of digestibility. Although there was a difference in the digestibilities of the oils it was so small that the absorption of rapeseed oil was not appreciably altered. Applying the method of Rice *et al.* (16) for measuring available calories, Middleton and Campbell were also unable to detect a difference between rapeseed oil and corn oil (17). As has been discussed, such findings are not in agreement with those of Carroll with rats of the Sprague Dawley strain. Under the conditions employed in this laboratory there appeared to be no practical difference in the utilization of rapeseed oil and corn oil. The significant nutritional characteristic of rapeseed oil, when compared with corn oil, was its effect on appetite, and was most evident in the early stages of the experimental period.

Acknowledgments

The authors wish to thank Miss Constance E. Cox and Mr. J. Claude Desloges for statistical and technical assistance respectively.

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THE EFFECT OF CARBON MONOXIDE ON OXYGEN CONSUMPTION, GLUCOSE UTILIZATION, AND GROWTH IN MAMMALIAN CELLS IN VITRO[1]

SAMUEL DALES AND KENNETH C. FISHER

Abstract

The effect of carbon monoxide on respiration, growth, and carbohydrate metabolism of Earle's L strain cells was investigated. The rate of *gas* uptake by suspensions of cells in a Warburg respirometer was the same from mixtures containing various proportions of $CO/O_2/N_2$ or containing only O_2/N_2. *Apparently* carbon monoxide was not inhibiting oxygen consumption. In respirometers filled with carbon monoxide and oxygen in the ratio of 9:1, illumination caused the net gas uptake to rise 23% above that of the controls. This suggests that the rate of oxygen consumption in the dark was reduced as a consequence of an inhibition of a respiratory enzyme by carbon monoxide. In keeping with this suggestion, a spectroscopic examination revealed the presence in L cells of absorption bands corresponding to those of the cytochrome enzymes. Using differential manometers it was established for the first time that carbon monoxide is taken up by L cells in the dark. It is concluded that a fraction of the *gas* uptake which is represented by the consumption of oxygen associated with normal metabolism must be reduced in the presence of carbon monoxide. It is also concluded that L cells oxidize carbon monoxide to carbon dioxide. The rate of multiplication of cells was reduced by carbon monoxide while the rate of glucose breakdown and lactic acid production were markedly accelerated, suggesting that there was an inhibition of cytochrome oxidase. Low partial pressures of oxygen also reduced the rate of multiplication of L cells while increasing the rate of glucose disappearance and the rate of lactic acid production. The observations reported here thus emphasize the role of aerobic oxidations in the maintenance of the maximum rate of growth.

Introduction

Warburg's (2) experiments with the effect of carbon monoxide on the consumption of oxygen by yeast cells led to his discovery of a light-dissociable reaction between carbon monoxide and a respiratory enzyme in yeast. Evidence for the occurrence of a similar enzyme system in many plant and animal tissues has since been obtained. In several species of bacteria (3), in *Chlorella* (4), and in various frog tissues (5), illumination has been shown to reverse either partially or completely the inhibition of oxygen uptake by carbon monoxide. A respiratory enzyme having characteristics similar to the yeast enzyme has also been found in heart muscle (6).

In organisms affected by carbon monoxide it was not only the oxygen uptake which was inhibited but also various physiological processes which are associated with it. In these instances illumination was also shown to reverse the inhibitory effects of carbon monoxide. Examples of such processes which showed inhibition and 'light-reversal' include division in yeast (7) and in sea-urchin eggs (8), the beating of embryo-fish hearts (9), the passage of impulses along nerves (10), and the development of insect pupae (11). The rates of glycolysis of both normal and malignant tissues, bone marrow cells, blood platelets, and leucocytes (7, 12) which had been increased by carbon monoxide

[1]Manuscript received September 9, 1958.
Contribution from Department of Zoology, University of Toronto, Toronto 5, Ontario.

were decreased upon illumination. The implication arising from this evidence is that carbon monoxide inhibited the various physiological activities of the organism by inhibiting the function of the respiratory enzyme.

It might be assumed from the foregoing that the uptake of oxygen and other physiological processes dependent upon oxygen consumption were always reduced in the presence of carbon monoxide. Actually, for example, growth in vitro of nerve cells (13) and of connective tissue cells (14) is not affected by carbon monoxide; and the uptake of gas by frog muscle in an atmosphere of oxygen and carbon monoxide was shown by Fenn and Cobb (15) to be greater, not less, than the uptake from air. Subsequent experiments by Schmitt and Scott (5), by Stannard (16), and by Clark, Stannard, and Fenn (17) on isolated tissues of frogs and rats and by Clark (18) on whole animals showed that in all these cases carbon monoxide was burned to carbon dioxide. Heart and skeletal muscles were found to take up gas at a markedly increased rate while liver, nerve, kidney, and testis of the frog and liver and tumor tissue of the rat showed only small increases in gas uptake (15). No stimulation of gas uptake was produced by carbon monoxide in muscle from the fish *Fundulus*, or in muscle from several species of invertebrates (19).

In any investigation, therefore, in which carbon monoxide is used to test the participation of the cytochrome respiratory enzymes in a given organism, it is necessary to be aware of the possibility that an oxidation of this test agent can occur. The importance of this possible oxidation may have been overlooked by Wainio and Cooperstein (20), when they suggested in a recent review on mammalian cytochromes that "carbon monoxide does not usually inhibit respiration of mammalian tissues." These authors apparently failed to recognize the possibility that the oxygen consumed in the oxidation of carbon monoxide might mask an inhibition of the normal oxygen consuming reactions.

Although the effect of carbon monoxide upon the metabolism of both plant and animal material has been investigated extensively, no studies on pure strains of mammalian cells grown in vitro have thus far been reported. The effect of carbon monoxide upon growth, oxygen consumption, and carbohydrate metabolism in L cells was, therefore, studied in order to obtain information regarding the respiratory mechanism in these cells and its association with growth.

Materials and Methods

Cell Strains

In these experiments we used Earle's L strain cells (21) derived from a single cell of mouse subcutaneous connective tissue, and strain MK II cells, a type that was derived from the monkey kidney and which had changed its morphological appearance (i.e. "transformed") in the laboratory of Dr. R. C. Parker (22).

Culture Methods

Cultures of these cells were grown at 38° C in one or other of synthetic media Nos. 635, 858, and 1066 (23, 24) supplemented in each case by the

addition of 20% horse serum. In media 858 and 1066 the purified coenzymes were, however, replaced by a coenzyme concentrate (Armour). The last medium differs from medium 858 mainly in that the fat-soluble vitamins and ferric nitrate are omitted and five B vitamins are added. From the present work there is no indication that the observations varied with the medium used.

The culture techniques employed were those developed by Earle and his associates (25), modified as described by Parker, Healy, and D. C. Fisher (26).

In studies on the rate of cell division, approximately 2×10^5 cells, contained in 0.1 ml, were delivered into "T-12" type flasks and to each was added 2.5 ml of nutrient medium. The flasks were incubated at 38° C for 96 hours. The number of cells present in the cultures was determined by dissolving the cytoplasm in 2% citric acid and counting the stained nuclei on the haemocytometer according to the procedure elaborated by Earle, Sanford, Evans, Waltz, and Shannon (27).

Analytical Procedures

Glucose content was determined by the method of Somogyi (28) and lactic acid by the procedure of Barker and Summerson as modified by Umbreit and others (29). Deproteinization was accomplished by using Somogyi's procedure which involves $ZnSO_4$ and $Ba(OH)_2$.

Preparation and Use of Gas Mixtures in Growth and Respiration Experiments

The usual practice of growing cell cultures in the presence of a gas mixture containing 20% O_2, 8% CO_2, and 72% N_2 was generally followed. The relatively high partial pressure of CO_2 maintains the pH of the nutrient medium below 7.5.

In some experiments the effects of CO on cultured cells were investigated. For these, commercially supplied oxygen, carbon dioxide, and nitrogen were used. Carbon monoxide was prepared by dropping 90% formic acid onto hot, concentrated sulphuric acid, passing the gas evolved through a column of soda lime to remove formic acid, and collecting the gas in bottles, over dilute alkali.

In order to gas the "T" flasks and at the same time maintain sterility in the medium, two No. 16 syringe needles were pushed through the rubber stopper of the flask and acted as inlet and outlet for the gas. Pressure tubing was attached to the outside of the needles, each tube ending in a glass bulb stuffed with a cotton plug. This apparatus was then sterilized.

Gas mixtures containing low partial pressures of oxygen were prepared by removing the desired quantity of oxygen from commercial tank N_2 (oxygen impurity about 2%). Copper filings heated to 500° C and/or an oxygen absorber described by Peters and Van Slyke (30) were used for this purpose.

Measurement of Respiration by Cultured Cells

The rate of O_2 consumption and CO_2 production were measured at 37° C by Warburg's indirect method (29) using 7-ml vessels. Either air or various proportions of CO and O_2 or N_2 and O_2 were used in the gas phase. The cells were contained in a total volume of 1.6 ml in each vessel.

As a suspension medium Earle's inorganic salt solution (31) buffered with 0.02 *M* phosphate, at pH 6.65–6.80, was used.

Determination of Carbon Monoxide Uptake

The uptake of CO was measured in Summerson differential manometers. Two such manometers (Fig. 1) are used. *One* vessel of *each* manometer is

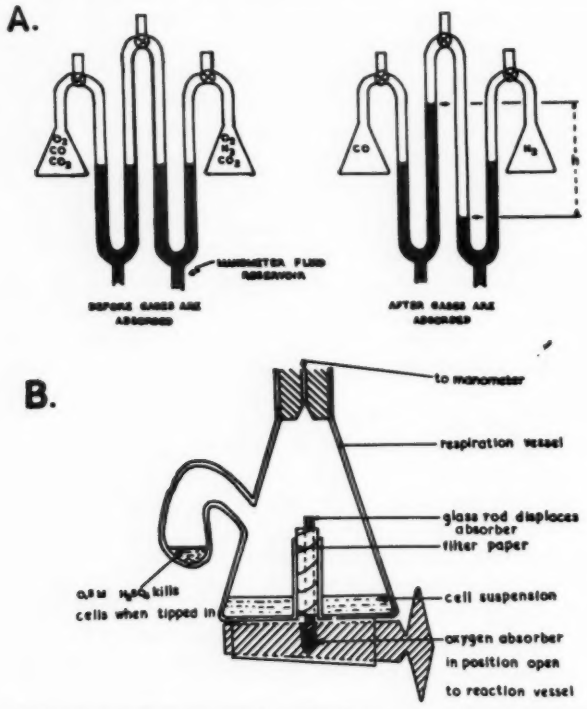

FIG. 1. A diagrammatic representation of a Summerson differential apparatus. A. Manometer and vessels are represented to show the change in manometer fluid levels after the O_2 and CO_2 are absorbed. B. The Dixon–Keilin (1) respiration vessel shown to illustrate the method by which oxygen absorber is admitted into the respiration chamber.

filled with a mixture of CO/O_2 and the *other* vessel with a mixture of N_2/O_2. The O_2 (and CO_2) in both vessels of *one* manometer is absorbed, as described below, at the beginning of the experiment. Since the oxygen concentration in the N_2/O_2 mixture is not exactly the same as in the CO/O_2 mixture, a small difference in pressure between the two vessels develops as a consequence of absorption of the oxygen. This difference is a measure of the initial difference in the partial pressure of N_2 and CO in the respiration vessels.

At the end of the experiment the O_2 and the CO_2 are absorbed in both vessels of the *other* manometer. A larger difference in pressure between the two vessels than that recorded in the first manometer, at the beginning of the experiment, is now observed. Hence either N_2 or CO must have been taken

up by the cells during the experiment. The cells do not take up N_2 from an atmosphere containing pure nitrogen and there are no data indicating that they take up gaseous nitrogen under any conditions. It is, therefore, inferred that the *change* in the pressure difference which developed as the experiment proceeded occurred because CO was taken up.

The Summerson differential manometers (32) were prepared as follows:

Two milliliters of cell suspension were added to each respiration vessel, of about 10 ml total volume. The average number of cells per vessel was 2.5×10^7. The side arm of the vessels contained 0.1 ml of H_2SO_4. Into each center well was placed a roll of filter paper and into the center of the roll was inserted a short glass rod. The well within the stopcock, which is placed underneath each reaction vessel, was filled with the hyposulphite (dithionate) oxygen absorber prepared by the method of Peters and Van Slyke (30) but containing twice the amount of each chemical. This was sealed off from the reaction chamber initially. By turning the stopcock at the desired moment the oxygen absorber was exposed to the reaction chamber (see Fig. 1).

After equilibration in the constant-temperature water bath, acid was tipped from the side arm of both vessels into the mainspace on one of a pair of manometers, thereby killing the cells. Several seconds later the stopcocks under both vessels were turned to expose the oxygen absorber to the reaction chamber.

Following the desired period of respiration the tissue was killed and the oxygen absorber admitted into the reaction chamber in the other manometer of the pair.

The hyposulphite used to absorb the oxygen is in an alkaline solution. It therefore took up not only the O_2 but also any CO_2 present or produced by adding acid to the cells. After admitting the O_2 absorber the only gas left, therefore, in one vessel of each pair was N_2 while the only gas left in the other was CO.

The change in the differential reading between the end and the beginning of the experiment represents the volume of CO taken up by the cells thus: $\Delta P_L - \Delta P_R = \Delta h_L - \Delta h_R$, when $(\Delta h_L - \Delta h_R)$ represents the difference in height of the manometer-fluid levels and ΔP is the pressure change calculated from the difference between Δh_L and Δh_R. The theoretical considerations upon the validity of which the calculated results are based have been dealt with by Summerson (32).

CO uptake by L cells was determined simultaneously with the measurement of O_2 consumption and CO_2 production and using aliquots of the same cell suspension.

Analysis for Low Partial Pressures of Oxygen

To establish the actual O_2 concentration gas samples were analyzed using a single-vessel Warburg manometer. After flushing of the Dixon–Keilin vessel with the gas mixture under analysis, the O_2 absorber was admitted into the center well. The O_2 content could be calculated from the resulting change in pressure.

Correction for CO Uptake by Potassium Hydroxide

Potassium hydroxide is used in the CO_2-absorbing solution. This alkali also absorbs some CO converting it into formate in proportion to both the CO partial pressure and the concentration of alkali (33). In some cases, therefore, weak alkali (0.5% KOH), which was shown not to absorb measurable amounts of CO, was used. In general, corrections were made when a stronger KOH solution was used.

The O_2 absorber also contains strong alkali. This did not interfere with the measurement of CO uptake, however, because the exposure of CO in the vessels to this solution was exactly the same in the vessels tested at the beginning of an experiment and in the other tested at the end.

Results

(*a*) *Gas Uptake at Various Partial Pressures of Carbon Monoxide*

The *gas* uptake in mixtures containing O_2/N_2 (column 4) on the one hand and O_2/CO, or $O_2/N_2/CO$ (column 8), on the other hand is presented in Table I.

TABLE I

Gas uptake by cultured cells in the presence of varying pressures of carbon monoxide

	Controls			Carbon monoxide				Mean difference	
	Composition of the gas phase, %			Composition of the gas phase, %					
Number of experiments (1)	O_2 (2)	N_2 (3)	10^{-6} mm³ O_2/cell/hr (4)	O_2 (5)	N_2 (6)	CO (7)	10^{-6} mm³ gas uptake/cell/hr (8)	Col.(4) − col.(8) with its standard error (9)	Probability of difference by *t* test for paired data (10)
3	10	90	2.27±0.45	10	80	10	2.05±0.44	0.22±0.190	0.1
3	10	90	2.17±0.43	10	40	50	2.10±0.39	0.07±0.017	.05
19	10	90	1.78±0.09	10	0	90	1.78±0.08	0.0 ±0.041	1.0
5	5	95	1.76±0.10	5	0	95	1.53±0.11	0.23±0.042	.01

NOTE: All values are from experiments with paired replicates. Values for gas uptake per hour are the averages of observations from experiments lasting 1½ hours. The standard errors are quoted in columns (4) and (8) to indicate the range of values from one experiment to another. Vessels were shaken 120 times per minute through an excursion of 2.5 cm. Experiments were conducted at normal indoor light intensities.

There is no significant difference, as indicated by the *t* test (column 10) between the *gas* uptake by L cells in the controls and the uptake in the presence of 10, 50, and 90% partial pressures of CO. There is, however, a small but significant decrease in gas uptake when the concentration of CO is increased to 95% (this decrease was observed in each of the five experiments). In similar experiments using MK II cells the results were generally the same as with L cells.

If the *gas* uptake actually measured is inferred to be *oxygen* uptake, then it might be concluded from Table I that partial pressures of CO ranging from 10% to 90% have no effect and 95% only a slight effect on the uptake of oxygen by cultured cells. That such a conclusion would be quite erroneous will be shown by the results below. It may be recorded here that the oxygen consumption of yeast was reduced in the $\frac{CO}{O_2} = 9$ mixture just as originally described by Warburg (2).

(*b*) *Effect of Illumination*

To test the effect of illumination on the gas uptake of L cells in CO a 4.5-amp carbon arc lamp was arranged to project a horizontal beam of light along the path through which the respiration vessels moved. This illumination was found to restore adequately the respiration of yeast inhibited by carbon monoxide.

The average uptake of gas by L cells in light and darkness when they were gassed with 90% CO/10% O_2, or with air was calculated from four separate experiments and is presented in the bar diagram in Fig. 2. In vessels contain-

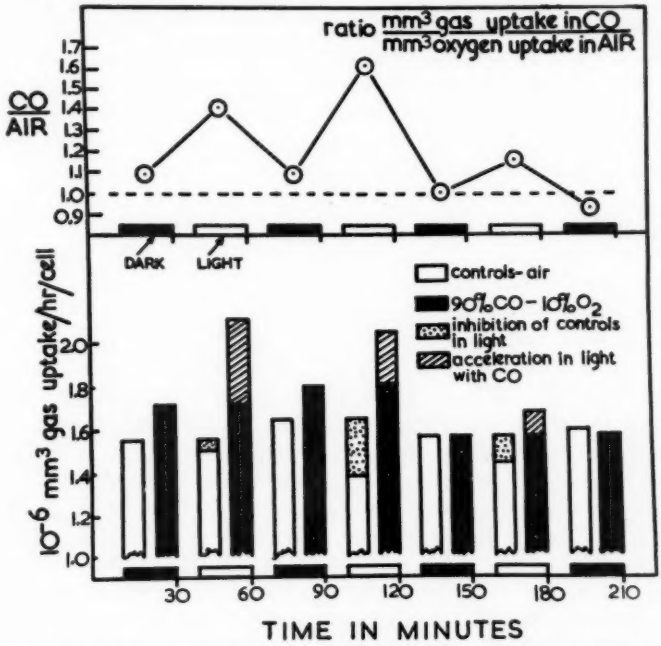

FIG. 2. Effect of illumination upon the gas uptake of L cells, when air is the gas phase in the controls and 90% CO/10% O_2 the gas phase in the experimental vessels. The ratio of gas uptake in the experimental vessels to that in the controls is shown above the bar diagram in order to emphasize the acceleration in gas uptake which occurs when the vessels with CO are exposed to light.

ing air the *oxygen* consumption during periods of illumination is invariably lower than in darkness. Light, therefore, must have some effect on the metabolic processes of which oxygen consumption is an indicator. In at least one report, beef retina tissue respiring in air had a higher rate of oxygen uptake in the dark than in light and respiration in white light was lower than in light of any color (34). Fenn and Cobb (15), on the other hand, found a stimulation, not an inhibition, of the uptake of O_2 by frog muscle respiring in air, when it was illuminated by 100-w lamps. In this instance, the relatively thick piece of tissue combined with a low light intensity may have produced

the conditions in which no injurious effects were present. It has been our experience that L cells respiring in air, when placed a foot or less from the arc lamp, took up progressively less oxygen with time, and this gradual diminution in O_2 uptake could not be reversed by returning the cells to darkness. Clearly, permanent changes were being produced.

It can be seen that in the presence of CO the gas uptake was increased during periods of illumination and fell off again when illumination ceased. The change in the ratio of (gas uptake in CO)/(gas uptake in air) is shown in the top panel of Fig. 2. It can be seen that this ratio was increased each time the cells were exposed to illumination and was reduced in each instance that illumination was stopped. Illumination, therefore, stimulated gas uptake by L cells when CO was present in the gas phase. If this increase in the rate of gas uptake upon illumination is strictly comparable to the similar increase which takes place in the case of yeast in the presence of CO, then it would have to be concluded that the cytochrome enzyme system is present in L cells, as it is in yeast. However, unlike the situation in yeast, the data for L cells in Table I do not show clear evidence of an inhibition of oxygen uptake by CO.

Further evidence concerning the presence of the cytochrome system was sought from a spectroscopic examination of the living cells. About 1×10^8 cells were centrifuged and suspended in a small volume (1.0 ml) of buffered Earle's solution. This suspension was placed in a chamber to form a column about 1 cm thick and the spectrum of the light transmitted through it was examined by a microscope-spectroscope. Two wide absorption bands were located in the region 550 mμ. These would appear to correspond to the bands at 566 and 550 mμ representing cytochromes *b* and *c*, which were found in absorption spectra of mammalian heart preparations (6). A third narrow band was located towards the green, presumably corresponding to the band at 520 mμ representing cytochrome *d* (*b* plus *c*), in these heart preparations. A distinct band in the 605 mμ region was not detected. It is inferred that cytochrome oxidase was present in only small quantity. After the addition of hydrogen peroxide (one drop of a 0.2% solution) no bands could be detected. They reappeared after a lapse of several minutes, and once again disappeared when the addition of peroxide was repeated. When, however, a few grains of sodium cyanide were added to the cell suspension the absorption bands were visible even upon the addition of peroxide. The oxidation of the cytochromes is apparently inhibited by cyanide.

It must be concluded that the failure of CO to reduce the rate of gas uptake by L cells is not due to the absence of the cytochrome enzyme system. The change in the gas uptake upon illumination, which is shown in Fig. 2, is probably due to an increase in the rate of oxygen consumption. These experiments using light were done with gas mixtures containing 90% CO and 10% O_2. In such mixtures, as Table I showed, there is no *apparent* effect of CO, since the rate at which gas is taken up in the presence of CO is the same as in its absence. It is thus necessary to conclude from experiments with illumination that CO in the dark did have an effect, that it was, in fact, inhibiting the

uptake of oxygen. These two observations can be reconciled only by concluding either that in the dark CO was itself taken up at a rate which just happens to have been equal to the decrease in the rate at which oxygen was taken up, or that CO causes nitrogen gas to be taken up. Reference has already been made to several instances in which CO was taken up by cells and this appears to be the more acceptable of the alternatives.

If we assume that an oxidation of CO can occur in L cells, then the inhibition of normal oxygen consumption by CO must be at least equal to

$$\frac{\text{increase in the rate of gas uptake in 90\% CO upon illumination}}{\text{rate of oxygen uptake in the controls during illumination}},$$

which in the experiments illustrated at the top of Fig. 2 averaged 23%.

(*c*) *The Uptake of Carbon Monoxide by L Cells*

The total gas uptake, the O_2 uptake, the uptake of CO and the production of CO_2 were measured on aliquots of the same cell suspension. Comparing columns (2) and (3) in Table II it is apparent, as it was in Table I, that the total gas uptake in the presence of CO nearly equalled the O_2 uptake in the absence of CO. When present, however, CO was taken up (column (10)). Evidently the normal O_2 consumption must have been reduced in the presence of CO by an amount nearly equal to the uptake of CO and any O_2 associated with this latter. Thus without the measurements of CO uptake it would appear that CO was not inhibiting oxygen consumption.

Two suggestions must be considered about the fate of CO taken up by L cells. It could be converted catalytically into formate ($CO + H_2O \rightarrow HCOOH$), a mechanism thought by Warburg (33) to occur in some cells, or it could be oxidized catalytically to CO_2 ($CO + \frac{1}{2} O_2 \rightarrow CO_2$).

To test whether CO was taken up without oxygen by L cells a gas mixture was prepared containing 90% CO/10% N_2. With this in the gas phase of a respiration vessel containing a suspension of L cells no change in volume occurred over a period of 2 hours. On admitting air into the respiration vessel, gas consumption was resumed, indicating that the cells had remained metabolically active. Oxygen is, therefore, required for the uptake of CO. The most plausible explanation for this observation must be that CO, on being taken up by L cells, is oxidized to CO_2.

For the 10.5 mm^3 of CO taken up (Table II, column (10)) to be oxidized to CO_2, as described above, 5.3 mm^3 of O_2 would be required. Of the total gas uptake of 72.1 mm^3 (in CO) only $(72.1-10.5-5.3)$ mm^3, i.e. 56.5 mm^3 could be O_2 consumed by the usual processes, while in the absence of CO 76.5 mm^3 of O_2 was taken up. In the presence of CO then, the O_2 consumed by the usual cellular processes was inhibited by $\{(76.5-56.5)/76.5\} \times 100 = 26\%$ approximately. This agrees satisfactorily with the estimate of 23% obtained from the illumination experiments described earlier.

From the equation given above it can be seen that the R.Q. for the oxidation of CO to CO_2 must be 2. The R.Q. of L cells in the absence of CO is, on the average, unity (see Table II, column (7)). Thus if a fraction of the total *gas*

TABLE II

Gas exchange in L cells in the presence and absence of carbon monoxide
$mm^3/12.5 \times 10^6$ cells in an experiment lasting 3½ hours

	Warburg, one vessel, manometer					Respiratory quotient			
Atmosphere in respiration vessel	90% N_2 10% O_2	90% CO 10% O_2	Probability of difference between (2) and (3) by t test for paired data	90% N_2 10% O_2	90% CO 10% O_2	90% N_2 10% O_2	90% CO 10% O_2	Probability of difference in R.Q. by t test for paired data	Differential manometer, mm^3 volume change during the experiment equals mm^3 of CO taken up by the cells
Gas measurement (1)	O_2 uptake (2)	Gas uptake (3)	(4)	CO_2 formed (5)	CO_2 formed (6)	(7)	(8)	(9)	(10)
	76.5	72.1	.06	77.7	76.6	1.01	1.24	.01	10.5
Standard error of mean	0.33	1.4		9.0	8.5	.02	.06		1.2

NOTE: Averages, with their standard errors, of nine separate experiments. In all but one of these experiments, the R.Q. in the presence of carbon monoxide was higher than in the controls. Experiments were conducted at normal indoor light intensities.

uptake in 90% CO resulted in an oxidation of the CO, it might be expected that the R.Q. of L cells, respiring in the presence of CO, would increase. It was estimated above that about 25% of the *gas* uptake could be accounted for by assuming that L cells oxidized CO. From this quantity and from the values of the R.Q.'s just mentioned the respiratory quotient of L cells respiring in the presence of 90% CO can be computed to be 1.09. The actual R.Q. observed in the presence of 90% CO was 1.24 (column (8)) and this was a significantly higher value (column (9)) than the 1.01 of the controls (column (7)). The direction of the increase is, therefore, in agreement with the presumption that some monoxide was oxidized to CO_2, though the amount of the increase is larger than expected.

(*d*) *The Effect of Carbon Monoxide on Growth and Carbohydrate Metabolism of L Cells*

To study the effect of CO on cell division and on glucolysis,* replicate cultures of L cells were prepared in "T" flasks and were gassed with either 70% CO / 20% O_2 / 10% CO_2 or with a corresponding gas mixture containing nitrogen instead of CO. The partial pressure of CO used could not be greater than 70% since it was desirable to maintain the same partial pressures of CO_2 and of O_2 in the experimental flasks as were present in the controls. Line 2 of Table III shows that the cell count increased by 11.2 times in the controls but

TABLE III

Effect of carbon monoxide on growth and glucolysis in L cells

Per ml of culture (1)	Control: 20% O_2/10% CO_2/70% N_2 (2)	Carbon monoxide: 20% O_2/10% CO_2/70% CO (3)
(1) Final cell count	729,900 ± 42,300	349,000 ± 9,700
(2) $\frac{\text{Final count}}{\text{Initial count (inoculum)}}$	11.2	5.4
(3) γ glucose disappearing	265 ± 36	768 ± 163*
(4) γ lactic acid appearing	54 ± 14	449 ± 29

*Based on eight observations.

NOTE: Averages and their standard errors of values obtained from nine individual cultures from three separate experiments (two, three, or four replicate cultures per experiment).

only by 5.4 times in CO. This partial pressure of CO, therefore, inhibited the growth but not completely. Carbon monoxide increased the glucose utilized in the cultures, and the lactic acid production, which was low in the controls, was increased by a factor of nearly 10. The increase in lactic acid formation accounts fairly closely for the increase in glucose utilization (i.e. 768 γ − 265 γ = 503 γ is close to 449 γ − 54 γ = 395 γ). In fact when these differences were computed for each single experiment the discrepancy, which averaged 108 γ, was not statistically significant.

*By 'glucolysis' is meant the conversion by the cells of glucose into lactic acid.

TABLE IV

Growth and glucolysis of L cells at low partial pressures of oxygen

Per ml of medium (1)	Experiment 1		Experiment 2	
	Control 20% O_2/8% CO_2/72% N_2 (2)*	0.5–1.5% O_2 10% CO_2/90% N_2 (3)*	Control 20% O_2/8% CO_2/72% N_2 (4)†	0.1% O_2 10% CO_2/90% N_2 (5)†
Cell count	647,100 ± 50,000	668,700 ± 137,600	774,700 ± 42,000	278,600 ± 2,500
Final count / Initial count (inoculum)	4.5	4.6	6.5	2.4
γ Glucose disappearing	220 ± 15	370 ± 29	360 ± 47	860 ± 9
γ Lactic acid appearing	37 ± 23	173 ± 42	93 ± 5	671 ± 13

*Average values and their standard errors from three separate experiments (three replicates each) of control cultures and from three separate experiments (two replicates each) of experimental cultures.

†Average values and their standard errors from two separate experiments comprising nine individual control and nine experimental cultures (four or five replicates per experiment).

(*e*) *Effects of Low Partial Pressures of Oxygen on Growth and Glucolysis of L Cells*

Two series of observations were made. In one series the partial pressure of oxygen in the gas mixture was between 0.5%–1.5%, in the other series it was only 0.1%. A comparison of columns (2) and (3) of Table IV shows that in flasks in which the O_2 partial pressure was approximately 1%, cells divided at the same rate as when the tension was 20%, although the low concentration of O_2 caused an increase in glucose disappearance and in lactate appearance. At 0.1% O_2 there was a large reduction in cell division and a very large increase in glucolysis, as is shown in columns (4) and (5) of Table IV. Under the conditions of these experiments, therefore, L cells require the presence of oxygen in order to maintain a normal rate of division.

As was the case with CO, in cultures incubated at low partial pressures of oxygen the increase in lactic acid, above that found in the controls, accounts closely for the larger amount of glucose disappearing from the medium. In 1% O_2 the glucose utilized was greater than that used in the controls by 150 γ. This quantity is not significantly different, in the statistical sense, from the increase in lactic acid production in 1% O_2, which amounted to 140 γ. Similarly in 0.1% O_2 the increased lactic acid production closely approximates the increase in glucose utilization.

Each culture of the type used to obtain the data of Tables III and IV contained initially about 900–1000 γ of glucose. It is apparent from column (5) of Table IV that under anaerobic conditions practically all of the glucose had disappeared. It is, therefore, possible that the increase in cell count may have been limited by insufficient glucose when the partial pressure of oxygen was low rather than by the low oxygen, per se.

Discussion

In their original paper Fenn and Cobb (15) suggested that the stimulation of gas uptake seen in the presence of carbon monoxide could result from the catalytic oxidation of this gas similar in mechanism to that shown with various colored haem compounds (35). The similarity between the iron-containing cytochrome enzymes and the haem compounds used by Negelein suggested to Fenn and Cobb that the cytochromes might be involved in the oxidation of carbon monoxide in living tissues. Later Clark, Stannard, and Fenn (17) and Clark (18) using radioactive tracers proved that in heart and skeletal muscle and in live mice and turtles, there was a conversion of CO to CO_2. The oxidation of carbon monoxide in nerve, skin, and other tissues was found to be negligible. From the amount of radioactive carbon dioxide produced, Stannard, Fenn, and Clark concluded that the oxidation of carbon monoxide was superimposed on a resting metabolism with, perhaps, a slight inhibition of the latter. A similar interpretation can be made of the results presented here, on the L strain mammalian cells. It appears that in the presence of carbon monoxide a fraction of the normal metabolism is inhibited, but there is also a concomitant uptake and probable oxidation of this gas.

More recently Breckenridge (36) showed beyond doubt that, in heart muscle, cytochrome oxidase was the tissue enzyme which had the property of a catalytic oxidation of carbon monoxide. This might also be the mechanism in frog muscle and in the L cell, used in the present investigation. It should, however, be noted that the presence of cytochrome oxidase does not always confer upon the cell the property of oxidizing carbon monoxide. Yeast, for example, certainly has cytochrome oxidase but it has not been shown to oxidize carbon monoxide. The ability to oxidize carbon monoxide which is apparently possessed by some cells argues that the cytochrome oxidases in different cells are different. Further evidence of this is to be found in the work of Castor and Chance (37), who describe small but distinct differences in the absorption spectra of cytochrome oxidase in yeast and ascites tumor cells, and in the work of Chance (38) where it is shown that there are differences in the kinetics of the reaction between carbon monoxide and cytochrome oxidase in yeast, bacteria, and muscle.

The observations on glucose utilization and lactic acid production reported in the present work emphasize the importance of carbohydrate degradation as the source of energy for growth. That glycolytic processes particularly may be the source of energy for cell division in embryonic and other rapidly growing tissue has engaged the attention of others. Thus, growth in vitro of chick fibroblasts (39) was not affected either by a lack of oxygen or by the application of respiratory inhibitors. Growth of these cells and also that of chick embryo midbrain (40, 41) was, however, inhibited by inhibitors of glycolysis. Other examples in which oxygen was apparently not required for optimal growth of cells in vitro can be found in the reports of Lazer (42), of Harris (14), and of Jones and Bonting (43).

Lipmann (44) found, however, that the presence of oxygen was necessary for the undiminished growth of connective tissue in vitro. Similar evidence supporting the view that some oxygen, if albeit at low concentrations, is necessary for cell division in chick embryonic tissue, in fibroblasts, and in sarcoma can be found in the results of Burrows (45), of Mottram (46), and of Wright (47). The last author did, however, obtain cell division and an outgrowth of the tissue in mouse carcinoma cells at O_2 partial pressures as low as 0.37–0.49% of an atmosphere.

From the evidence available it appears that cell division in the various cell types which have been studied is affected by the lack of oxygen in a varying degree. Reduction of the partial pressure of oxygen in the atmosphere in which the L strain cells were cultured reduced the amount of growth but this reduction could have resulted from an exhaustion of the carbohydrate of the medium rather than from a lack of oxygen. There is, therefore, a strong possibility, as suggested also by the results of Harris (14), that L cells are facultative anaerobes, able to grow anaerobically as long as the energy available to the cells from the glycolytic processes is adequate.

Acknowledgment

We wish to record that our research has been materially assisted by grants from the Ontario Cancer Research and Treatment Foundation, the National Cancer Institute of Canada, the Banting Foundation, and the University of Toronto's Cancer Committee. In addition we are most appreciative of the assistance of Dr. Raymond Parker of the Connaught Medical Research Laboratory in connection with the maintenance of stocks and culture of cells. We wish to thank also our assistant Miss Rae Shepard.

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THE BREAKDOWN OF ADENOSINE PHOSPHATES IN EXTRACTS OF XANTHOMONAS PHASEOLI[1]

R. M. HOCHSTER AND N. B. MADSEN

Abstract

Cell-free extracts of *Xanthomonas phaseoli* (XP8) can convert ATP to hypoxanthine, the chief intermediates being ADP, AMP, IMP, and inosine. The following enzymes concerned in the breakdown of ATP are present: adenosine triphosphatase, myokinase, adenylic acid deaminase, a 5′-nucleotidase acting on IMP, and a nucleosidase which converts inosine to hypoxanthine. It is concluded that the conversion of ADP to AMP is, for the most part, carried out by the myokinase, the direct dephosphorylation of ADP being of questionable significance in these extracts.

AMP is not dephosphorylated directly but is first deaminated to IMP. A method is presented which allows enzymatic deamination to be studied by manometric means. The deamination of AMP is found to explain the biphasic nature of the carbon dioxide output and uptake observed when hexokinase activity of extracts is measured by the manometric method.

Introduction

As part of a study previously published (1) on the mechanism of glucose-6-phosphate oxidation by cell-free extracts of the phytopathogenic organism *Xanthomonas phaseoli* (XP8), experiments were also carried out to determine the possible role played by hexokinase in such preparations. Although the participation of hexokinase in the glucose metabolism of this organism seemed likely (2), attempts to obtain confirmatory evidence by a manometric technique (3) led to the discovery of a biphasic reaction sequence the nature of which was not understood. This effect was subsequently traced to the presence of added ATP[2] or to its breakdown products. Exploratory studies on the nature of the oxidative phosphorylation system in cell-free preparations from *X. phaseoli* were hampered by the presence of enzymes which rapidly dephosphorylated adenosine nucleotides. This was further accentuated by the fact that only about 50% inhibition could be attained by the addition of high concentrations of fluoride. It soon became apparent that a thorough study of the breakdown of ATP might provide common answers to both of the above problems. It is the purpose of this paper to present the results of these efforts.

Detailed studies on the breakdown of ATP appear to have been carried out only with mammalian tissues (4, 5, 6, 7) so far. A search of the literature has not revealed a similar detailed study of these steps in microorganisms, although numerous reports exist on the release of inorganic phosphate from ATP (8, 9, 10, 11). Furthermore, myokinase activity (12), nucleoside breakdown and deaminase action (13, 14, 15, 16) have been discussed individually for a

[1]Manuscript received December 19, 1958.
Contribution No. 478, from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

[2]The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate (adenylic acid); IMP, inosine monophosphate; TPN, triphosphopyridine nucleotide; GSH, glutathione; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; P_i, inorganic orthophosphate.

variety of organisms but no comprehensive picture exists at present of the metabolic route involved in ATP breakdown in a single species.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ATP, ADP, AMP, adenosine, adenine, IMP, TPN, and inosine (Pabst Brewing Co.); hypoxanthine (Nutritional Biochemicals Corp.); D-glucose, D-ribose, D-xylose, D-arabinose, and D-lyxose (Pfanstiehl Laboratories Inc.); GSH (Schwarz Laboratories); and Tris (Sigma Chemical Co.).

Enzyme Preparations

X. phaseoli (XP8) was grown in a medium consisting of 1% yeast extract and 2% glucose. Fifty milliliters of this medium was inoculated from a yeast–glucose–$CaCO_3$ slant culture and incubated for 18–24 hours at 26° C on a rotary shaker. The inoculum thus obtained was then added to 700 ml of the medium contained in a 2800-ml Fernbach flask and again incubated in the same manner. Cells were harvested by centrifugation at 4000 r.p.m., washed twice with 0.9% NaCl, and suspended in two volumes of 0.1 *M* tris buffer (pH 7.7) containing 0.01 *M* GSH. Each batch of cells was tested for strain purity by the specific phage test (17).

The "whole extract" was prepared by treatment of the cell suspension in a Raytheon 200-w, 10-kc oscillator for 20 minutes at a power output of 1.0 amp, followed by centrifugation at 15,000×*g* at 5° C for 10 minutes. The supernatant solution was then dialyzed for 1 hour against running tap water at 10° C and for an additional hour against distilled water at 0–4° C.

The "particle-free extract" was prepared by treatment of the cell suspension in the oscillator for 5 minutes, followed by centrifugation for 90 minutes at 100,000×*g* in a Spinco model L preparative ultracentrifuge. The clear supernatant was poured off from the sedimented particles and cells, and was used directly.

Enzyme Assays

Hexokinase was determined by the manometric technique of Colowick and Kalckar (3) and since the organism was shown previously to contain a high concentration of glucose-6-phosphate dehydrogenase (1), the spectrophotometric method using the measurement of TPN reduction at 340 mμ (18) was also used.

Analytical Methods

Studies on the breakdown of nucleotides were carried out by incubation of the respective reaction mixtures at 30° C. Reactions were stopped by the addition of TCA to a final concentration of 5%. The mixtures were chilled and centrifuged and the inorganic phosphate was determined directly in aliquots of supernatant solutions by the method of Fiske and SubbaRow (19). Labile phosphate was determined as the inorganic phosphate released during 7-minute hydrolysis at 100° C in 1 *N* mineral acid. The remainder of the

supernatant solution was extracted three times with two volumes of diethyl ether to remove the TCA. Aliquots were then spotted on sheets of Whatman No. 1 filter paper together with suitable known reference compounds and subjected to ascending chromatography for 18 hours in a solvent system (at pH 3.7) consisting of isobutyric acid – concentrated ammonia – water in the ratio of 66:1:33 (designated as "System No. I" in Circular No. OR-10 published by Pabst Laboratories). Under our conditions the nucleotides of interest in the present study had the following R_f values: ATP and IMP, 0.23; ADP, 0.35; AMP and inosine, 0.52; hypoxanthine, 0.63; adenosine, 0.84; adenine, 0.92. After chromatography and drying of the papers the compounds were located according to their absorption when exposed to a Mineralight (model SL2537, with short-wave ultraviolet filter) in a dark room. Squares of paper (3 cm^2) containing the compounds in question were placed in 3-ml quantities of 0.05 *M* tris buffer (pH 7.7), and the mixtures shaken for 2 hours at room temperature. The buffered solutions were then analyzed spectrophotometrically (a similar piece of clear paper was also eluted and served as the blank). A molar extinction coefficient of 16,000 at 259 mμ was used to calculate the concentrations of the adenine compounds and 12,200 at 249 mμ for the inosine compounds. Where adenosine and inosine compounds had the same R_f, a modification of Smillie's method (7) was used, in which:

$$10^4 C^{\mathrm{I}} = 1.24 D_{240} - 0.54 D_{265},$$
$$10^4 C^{\mathrm{A}} = 0.90 D_{265} - 0.53 D_{240},$$

where C^{I} is the concentration of the inosine compound, C^{A} is the concentration of the adenosine compound, D_{240} and D_{265} are the optical densities at 240 and 265 mμ.

Paper electrophoresis was employed in cases where it was desirable to separate compounds which moved as one spot in the above ascending method. The apparatus was similar in design to that described by Smith (20). The compounds (dissolved in distilled water) were applied as spots (5λ) to Whatman No. 1 Chromatography paper (14×45 cm), previously saturated with 0.05 *M* citrate buffer at pH 3.5. A voltage gradient of 40 v/cm was applied for 70 minutes. Cooling was provided by a layer of carbon tetrachloride which was placed above the horizontally positioned paper strip. Nucleotides separated well under these conditions (ITP, 20.5 cm; ATP, 17.1 cm; ADP, 15.1 cm; IMP, 13.7 cm; AMP, 7.3 cm) but inosine and hypoxanthine were indistinguishable (2.3 cm).

Protein concentration was estimated by the spectrophotometric method of Warburg and Christian (21).

Experimental and Results

Hexokinase, Myokinase, and Adenylic Acid Deaminase as Determined by Manometric Methods

Experiments with cell-free, whole extracts of *X. phaseoli* revealed that glucose was oxidized readily and rapidly (at pH 7.2) provided Mg^{++} and sufficient ATP were supplied. When the manometric method of Colowick and

Kalckar (3) for the study of kinase activity was used, it was found (Fig. 1A, curve 1) that CO_2 evolution took place as soon as ATP was added from the side arm of the Warburg flasks. Contrary to expectations this gas output ceased after 6 minutes and was immediately followed by an apparent gas uptake. The biphasic nature of this reaction suggested that a second reaction, probably competing for the CO_2, was also taking place. At the peak, only 34.6% of the theoretical CO_2 evolution had been accounted for. The same result was obtained when the experiment was done in the presence of added fluoride (0.033 *M*). That the first stage of the reaction was truly a measure of the presence of hexokinase activity was proved by means of a spectrophotometric experiment, illustrated in Fig. 1B. It represents the absorption at

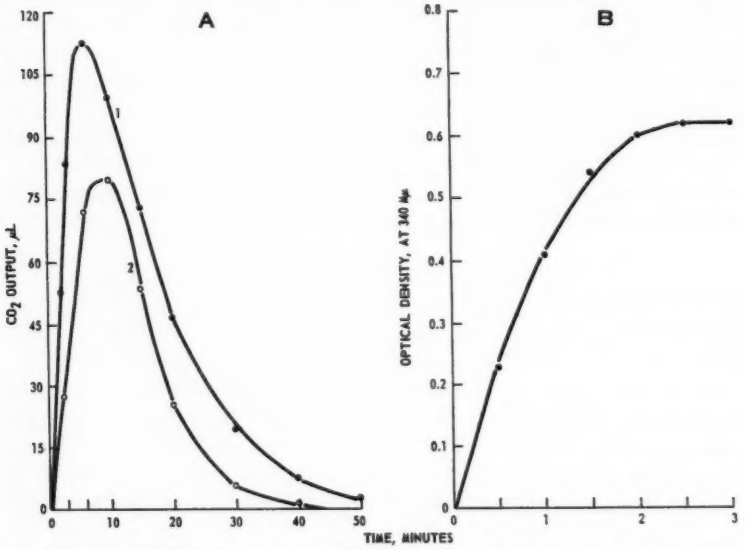

FIG. 1. Net hexokinase activity of *X. phaseoli* extracts as measured by manometric (A) and by spectrophotometric (B) means.

Vessel contents in experiment A: $NaHCO_3$, 60 µmoles; $MgCl_2$, 10 µmoles; whole extract, 1.0 ml, containing 40 mg protein; glucose (where used), 10 µmoles; ATP or ADP, 15 µmoles. Temperature: 30° C; gas phase: 95% N_2+5% CO_2; total volume: 3.0 ml.

Cuvette contents in experiment B: glucose (where used), 5 µmoles; glycyl glycine buffer pH 7.8, 100 µmoles; $MgCl_2$, 10 µmoles; ATP, 15 µmoles; whole extract, 0.05 ml (2 mg protein). Total volume: 3.0 ml; 0.3 µmoles TPN added at zero time.

340 mµ resulting from the addition of 0.3 µmoles TPN to a mixture containing glycyl glycine buffer, $MgCl_2$, glucose, ATP, and extract (a corresponding cuvette from which glucose was omitted served as the blank). In view of the previously demonstrated content in such extracts of a highly active glucose-6-phosphate dehydrogenase and of the absence of pyridine nucleotide transhydrogenase (1) the reaction described represents TPNH formation in the "Zwischenferment" reaction in which glucose-6-phosphate is the substrate. Thus, hexokinase is present in our extracts.

Since ATP acts as the phosphate donor in the hexokinase reaction and ADP does not (22), the latter was also used in the manometric experiment with the expectation that no reaction would be observed. But, as shown in Fig. 1A, curve 2, ADP also led to a rapid initial CO_2 output with a maximum at 10 minutes followed by a CO_2 uptake. This result is taken to indicate that the extracts contained a highly active myokinase which first catalyzed the formation of ATP + AMP from the ADP added. More direct evidence for myokinase is presented in a later section of this paper. The CO_2 uptake portion of the biphasic reaction sequence occurred with both ATP and with ADP and since both nucleotides gave rise to AMP (via myokinase) it was considered possible that the CO_2 uptake in the experimental system was caused by the further metabolism of AMP.

An experiment was then set up in which AMP served as the substrate and in which conditions were identical with those used in the manometric determination of hexokinase. The results show (Fig. 2, curves 1 and 2) that an immediate CO_2 uptake took place when AMP was tipped in at zero time in the presence of the extract. There was no gas exchange when the extract was omitted from the system or when a boiled extract was used. At this point it seemed most probable that the reaction observed was due to the deamination of AMP. This was further confirmed by the finding of a strongly positive Nessler reaction in aliquots from TCA-treated reaction mixtures and by the detection of relatively large amounts of IMP on papers containing such aliquots which had been subjected to electrophoresis (see "Materials and Methods") for 70 minutes. AMP (7.3 cm) was found to give rise to two new spots moving 13.7 cm and 2.3 cm. The former was identified as IMP and the latter as a mixture of inosine and hypoxanthine. Also, IMP, when used as "substrate" (Fig. 2, curve 3) did not cause any CO_2 uptake in the manometric system. In this connection it is interesting to note that the extracts used contained a highly active adenosine deaminase (Fig. 2, curves 4 and 5) which deaminated adenosine readily but not adenine (curve 6). Inosine and hypoxanthine were identified as the products by chromatography in the isobutyric acid – ammonia – water system.

Inasmuch as the above technique represents a new method for measuring the time course of an enzymatic deamination reaction it would be desirable to have data available with the aid of which more precise stoichiometric relationships could be ascertained. If CO_2 uptake is calculated on the basis of 1 mole per mole of ammonia released, then the values attained (Fig. 2) represent a deamination equivalent to 15 μmoles of substrate. This is 25% below the expected maximum. It is difficult at present to interpret such a result because in the presence of $NaHCO_3$-buffer and CO_2 the ammonia released can form NH_4HCO_3, probably some $(NH_4)_2CO_3$, and possibly also some $NH_4NH_2CO_2$ (23). The problem is further complicated by the fact that a slight rise in the pH of the reaction mixture takes place (0.1 unit). This will undoubtedly lead to an increase in the solubility of the CO_2 of the atmosphere in the vessel resulting in a "net uptake". On the basis of these considerations

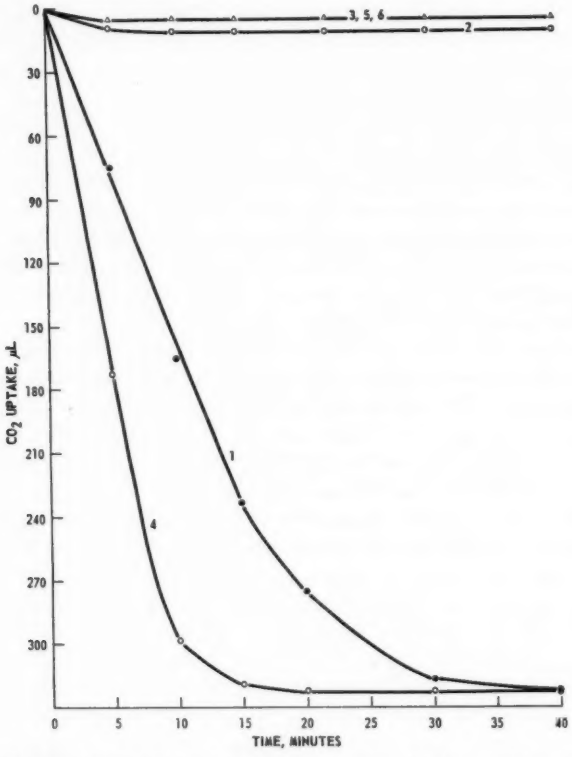

FIG. 2. Manometric determination of enzymatic deamination reactions (net values). Curve 1 = AMP, 2 = AMP without extract or with boiled extract, 3 = IMP, 4 = adenosine, 5 = adenosine without extract or with boiled extract, 6 = adenine.
Vessel contents: $NaHCO_3$, 60 µmoles; $MgCl_2$, 20 µmoles; whole extract, 0.5 ml, containing 20 mg protein; AMP, IMP, adenosine, or adenine, 20 µmoles. Temperature: 30° C; gas phase: 95% N_2+5% CO_2; total volume: 3.0 ml; initial pH: 7.8.

no attempt is made here to ascribe absolute stoichiometric significance to the values observed.

The Breakdown of Nucleotides as Measured by More Direct Analytical and Spectrophotometric Procedures

Incubation of nucleotides with extracts resulted in the release of inorganic phosphate. As Table I shows, the rate of P_i formation decreased in the order: ATP > ADP > IMP > AMP. Experiments I, II, and III were conducted with different extracts. The increased rate of P_i release from IMP as compared to AMP is slight but consistent and, as will be shown later, is significant with respect to the sequence of reactions. Determinations of the pH optima of nucleotide dephosphorylation showed ATP to have an optimum at pH 8.0 (normal curve), ADP to be between 7.5–8.0 (skewed slightly toward the lower pH's), and AMP to have a very broad (flat) optimum between pH 7.0–8.0

TABLE I

Formation of inorganic phosphate from nucleotides

Experiment No.	Tube	Substrate added	P_i formed (μmoles/15 min/mg protein)
I	*a*	10.0 μmoles AMP	0.56
	b	7.5 μmoles ADP	1.17
	c	7.5 μmoles ATP	1.58
II	*a*	10.0 μmoles AMP	0.47
	b	10.0 μmoles IMP	0.57
III	*a*	20.0 μmoles AMP	0.53
	b	20.0 μmoles IMP	0.62

NOTE: In addition to the nucleotide each reaction mixture contained 100 μmoles of tris buffer (pH 7.7), $MgCl_2$ equal to the amount of nucleotide, particle-free extract containing 6 mg of protein in experiments I and II, and whole extract containing 22 mg of protein in experiment III. Total volume: 3.0 ml. Incubation temperature: 30° C.

(skewed markedly toward the lower pH's). Subsequent studies were, therefore, conducted at pH 7.7.

The results of a detailed analysis of the time course of ATP degradation are given in Fig. 3. It is readily seen that the release of inorganic phosphate in the first 5 minutes is balanced by the decrease in acid-labile phosphate. Furthermore, in the first 2 minutes of reaction, the appearance of 4 μmoles of P_i was accompanied by the formation of 3 μmoles of ADP and of 1 μmole of AMP as well as by the disappearance of 4 μmoles of labile phosphate. [It is necessary to consider the part played by the highly active myokinase (see below) in any correlation of stoichiometric data.] These results may be taken as evidence that the first step in the degradation of ATP is the hydrolysis of the terminal phosphate grouping. The subsequent formation with time of IMP and of hypoxanthine is also apparent from Fig. 3.

It was more difficult to determine the fate of the ADP formed by enzymatic hydrolysis of ATP. The data in Table II show that the *X. phaseoli* extracts

TABLE II

The breakdown of ADP

Compounds found	Quantities (μmoles) at various times of incubation			
	0 min	2 min	5 min	10 min
Net inorganic phosphate	—	2.8	6.1	13.0
ADP	38.0	14.5	11.4	7.7
ATP	0.0	13.4	12.5	9.4
AMP	3.4	12.4	9.4	5.7
IMP	0.0	3.1	6.2	10.0
Sum of inosine+hypoxanthine	0.0	1.9	2.9	5.0
K_{equil} of myokinase reaction*	—	0.79	0.90	0.90

NOTE: Reaction mixture contained 40 μmoles of ADP, 40 μmoles of $MgCl_2$, 200 μmoles of tris buffer (pH 7.7), and 1.0 ml of whole extract. Total volume: 6.0 ml. Incubation temperature: 30° C.
*$K_{equil} = [(ATP)(AMP)]/(ADP)^2$.

contained an extremely active myokinase (cf. also the manometric data of Fig. 1A, curve 2). This reaction approached equilibrium in the first

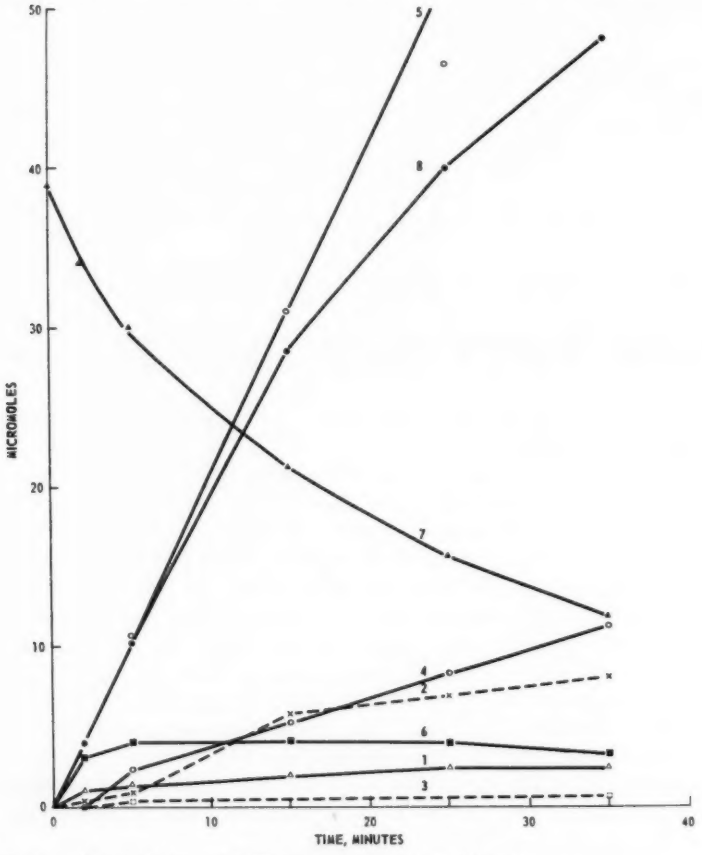

FIG. 3. The breakdown of ATP by an extract of *X. phaseoli*.
Curve 1 = AMP, 2 = IMP, 3 = inosine, 4 = hypoxanthine, 5 = net inorganic phosphate, 6 = ADP, 7 = ATP, 8 = decrease in acid-labile phosphate.
Contents of reaction mixture: ATP, 40 μmoles; $MgCl_2$, 50 μmoles; tris buffer (pH 7.7), 100 μmoles; particle-free extract, 1.5 ml. Temperature: 30° C; total volume: 2.5 ml.

2 minutes following incubation with ADP. In this brief period, 23.5 μmoles of ADP were dismuted while only 2.8 μmoles of P_i were released. The results suggest that the major portion of the ADP was transformed into ATP + AMP. It was not possible to determine whether any direct dephosphorylation of ADP occurred in view of the experimental difficulties raised by the presence of such high myokinase activity.

Incubation experiments with AMP as the substrate show (Fig. 4) that the rate of deamination of AMP (calculated as the sum of P_i + IMP) exactly equalled the rate of disappearance of AMP and was almost twice the rate of formation of P_i. Furthermore, not a trace of adenosine or adenine could be detected at any selected sampling time in this or other similar incubation

mixtures containing IMP, ADP, or ATP as substrates. The results in Fig. 4 also show that initial rates of formation occurred in the order: IMP (curve 2) → inosine (curve 3) → hypoxanthine (curve 4). The same order of appearance of the products of AMP utilization was obtained when the analysis was preceded by paper electrophoresis followed by elution of the respective areas. It has already been pointed out that the initial rate of P_i release from IMP was consistently greater than when AMP was the starting material. It is concluded, therefore, that AMP is not dephosphorylated directly but that it is first deaminated to IMP which is subsequently dephosphorylated to inosine. The latter is then converted to hypoxanthine after a short lag period. Theoretically, a net formation of ribose should occur during the conversion of inosine to hypoxanthine. Therefore, chromatograms of samples of a reaction mixture (similar in content to that described for Fig. 4) taken at 0, 15, and

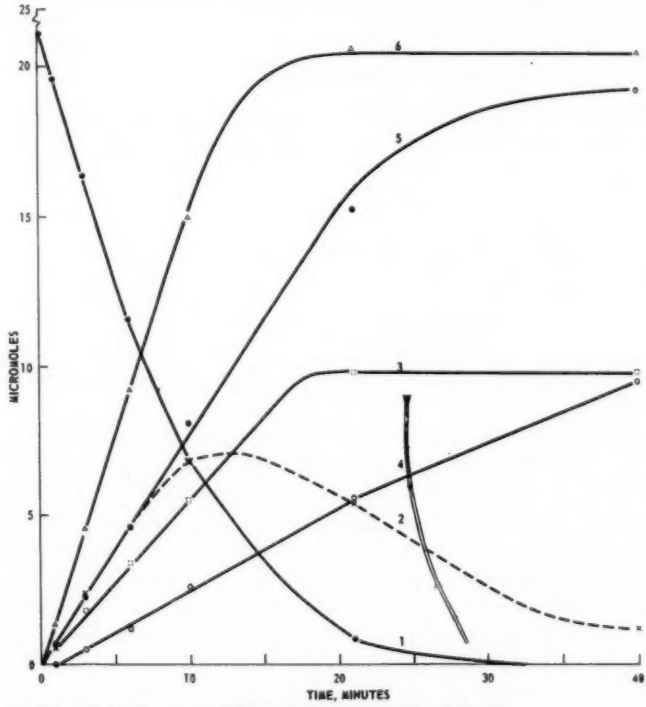

FIG. 4. The breakdown of AMP by an extract of *X. phaseoli*.
Curve 1 = AMP, 2 = IMP, 3 = inosine, 4 = hypoxanthine, 5 = net inorganic phosphate, 6 = deamination of AMP (calculated as the sum of P_i+IMP).
Contents of reaction mixture: AMP, 20 μmoles; $MgCl_2$, 20 μmoles; tris buffer (pH 7.7), 100 μmoles; whole extract, 0.5 ml. Temperature: 30° C; total volume: 3.0 ml.

30 minutes of incubation and developed with isobutyric acid – ammonia – water were sprayed with *o*-amino biphenyl (24). A bright red spot (characteristic of aldopentoses) was detected (R_f 0.42) in each sample, the intensity

rising with increasing time of incubation. Areas corresponding to this spot were cut out from another chromatogram, eluted with a minimum of water, and the eluate transferred to paper strips. Samples of authentic pentoses (ribose, xylose, arabinose, lyxose) were used as markers. After 70 minutes of electrophoresis in 0.05 *M* borate buffer (pH 9.2) with a voltage gradient of 40 v/cm, the unknown substance had travelled a distance of 9.5 cm from the origin. This corresponded exactly to the distance travelled by ribose (9.5 cm) and was quite different from the three other aldopentoses, which travelled a distance of from 11.4 to 12.9 cm. It also gave a strongly positive orcinol reaction (25) with an absorption maximum at 670 mμ. The sugar formed during the conversion of inosine to hypoxanthine is, therefore, identified as ribose.

Incubation of IMP with extracts followed by chromatographic analysis of samples taken at different times showed, apart from some unchanged IMP, only inosine and hypoxanthine.

Discussion

The pathway of ATP breakdown in extracts of *X. phaseoli* is presented in Fig. 5, in which solid arrows denote reactions for which positive evidence has

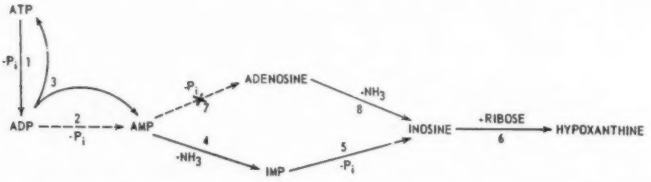

FIG. 5. Schematic presentation of the pathway of ATP breakdown catalyzed by cell-free extracts of *X. phaseoli*.

been presented. The over-all scheme shows considerable similarity to that found in rat brain homogenates (7), with a few exceptions. Smillie (7) found some adenosine in his reaction mixtures, suggesting that reaction 7 may occur in brain tissue, whereas we conclude that it is not formed at all under our experimental conditions. Moreover, Smillie found that AMP was almost quantitatively converted to inosine which accumulated at the end of his experiments. Our results, on the other hand, show that inosine is readily converted to hypoxanthine with the concomitant splitting off of ribose. After 40 minutes of reaction equal concentrations of inosine and of hypoxanthine were present in the *X. phaseoli* system.

The release of a molecule of ribose per molecule of ATP initially utilized is an interesting phenomenon which may have other implications and may bear upon the problem of the infectivity of the organism. Similarly, the high ATP-ase activity (relatively fluoride insensitive) found in our cell-free extracts may be of significance in the host–parasite relationship with which these studies will ultimately be concerned. Our lack of success in observing the net uptake of inorganic phosphate during oxidation of substrates by these extracts is

attributed to this high ATP-ase activity. Work is now in progress to discover an experimental approach which will allow the determination of P:O ratios without resorting to the indirect methods which must now, of necessity, be used with our extracts.

Our experiments have also indicated that the direct dephosphorylation of ADP (reaction 2) is probably not significant in view of the high myokinase activity. We have not succeeded, however, in eliminating this reaction entirely. Other workers (5, 7) have referred to similar difficulties.

Acknowledgments

The valuable assistance given by Mrs. C. Nozzolillo and by Miss M. Chisholm is hereby gratefully acknowledged.

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THE EFFECTS OF SARIN AND ATROPINE ON THE RESPIRATORY CENTER AND NEUROMUSCULAR JUNCTIONS OF THE RAT[1]

W. C. STEWART

Abstract

Records were made of the contractions of a slip of diaphragm muscle which was isolated from the circulation of the rat, while the nerve supply was preserved. Simultaneous records were also made of the contractions of the opposite (circulated) hemidiaphragm, of respiratory rate, of the Hering-Breuer reflex, and of contractions of the gastrocnemius muscle in response to stimulation of the sciatic nerve.

Low doses of sarin caused immediate respiratory arrest, purely central in origin; respiration was restored to normal at once when a large dose of atropine was given. When atropine was injected before sarin, much higher doses of sarin were required to depress the respiration, and now the respiratory paralysis took place at the neuromuscular junctions in the diaphragm, the respiratory center remaining relatively unaffected. It was concluded that respiratory paralysis by sarin could be purely central, purely peripheral, or both central and peripheral, depending on the doses of sarin and atropine employed.

Introduction

Sarin (isopropyl methylphosphonofluoridate) is a highly toxic cholinesterase inhibitor. The cause of death from sarin poisoning in animals, including the rat, is respiratory arrest (2). The respiratory arrest could be due to a direct effect of cholinesterase inhibition at synapses in the respiratory center, or to peripheral neuromuscular blockade in the respiratory muscles, or to both causes. In order to investigate the relative importance of central and peripheral mechanisms in the respiratory depression caused by sarin, it was necessary to devise a technique by which the effect of sarin on the respiratory center, and on neuromuscular conduction, could be separately and simultaneously observed. This question has been investigated, by deCandole *et al.* (2) for sarin in various species, and by Douglas and Matthews (1) for TEPP in cats, by recording the nerve impulses in the phrenic nerve.

In the present experiments the output of motor nerve impulses from the respiratory center was registered by the contractions of a section of diaphragmatic muscle, which had been deprived of the normal blood supply and so preserved from any neuromuscular blocking action of the sarin injected into the animal.

Methods

Operating Procedure

Rats (200–280 g) were anesthetized with Dial–urethane injected intraperitoneally. A tracheal cannula was tied in, and artificial respiration started;

[1]Manuscript received October 25, 1958.
Contribution from the Physiology Section, Defence Research Board of Canada, Suffield Experimental Station, Ralston, Alberta, as Suffield Technical Paper No. 99. (D.R.B. Project No. D52-89-50-15.)

the usual cycle of artificial respiration was 14 seconds of inflation with pure oxygen at a pressure 10 cm of water above atmospheric pressure, followed by 14 seconds during which the lungs were allowed to discharge to the atmosphere. The left jugular vein was cannulated, for intravenous injections. A ligature was tied to the left tendo achilles, for recording the contractions of the gastrocnemius muscle, and silver electrodes were applied to the corresponding sciatic nerve through an incision in the thigh.

An opening was made in the left chest wall by removing most of ribs 7, 8, and 9 and by cutting the sternal ends of ribs 4, 5, and 6, which were then retracted toward the head of the rat. This exposure gave a free approach to the left phrenic nerve and the left half of the diaphragm. A section of diaphragm about 10 mm wide was isolated by cuts parallel to the muscle fibers, one on each side of the point of entrance of the phrenic nerve into the muscle. The costal end of this slip of muscle was ligatured and cut away from the ribs, leaving the muscle attached to the ligature. The slip was gently retracted toward the head of the rat, exposing the abdominal side of the diaphragm and the large blood vessels in the central tendon. These vessels were cut between two ligatures, one of which remained attached to the slip, and the other to the central tendon. The latter was used later for recording the movements of the intact circulated half of the diaphragm. The isolated muscle slip was immediately placed in a small organ bath which had been molded of cotton gauze impregnated with collodion, to fit the opening in the chest wall. The phrenic nerve was carefully preserved. The bath was irrigated by a slow drip of a saline solution containing rat blood (per liter, NaCl 7.0, KCl 0.14, $CaCl_2$ 0.16, $NaHCO_3$ 0.5, glucose 2.0, heparin sodium 1000 units, blood obtained by decapitating one rat). The rat blood was added so that the sarinase (fluorophosphatase) present in the blood would destroy any traces of sarin which might accidentally contaminate the bath. The bath was stirred, oxygenated, and maintained at pH 7.4 by a stream of gas bubbles (95% O_2, 5% CO_2).

Recording Procedure

One end of the isolated diaphragm slip was attached to a hook which was embedded in the bottom of the organ bath: the ligature on the other end of the muscle slip passed over a system of pulleys, and was attached to a light spring-loaded lever. The movements of the lever were recorded on smoked paper. Similar arrangements were used to record the contractions of the intact hemidiaphragm and the gastrocnemius. Tetanic contractions of the gastrocnemius were recorded at intervals of 2.5 minutes by stimulating the sciatic nerve with a burst of square waves of 10 volts amplitude and 40 cycles per second frequency, the burst lasting about 20 seconds. These bursts were applied automatically by means of a cycle timer.

The respiratory rate was also recorded by means of an event timer similar in principle to that described by Gaddum and Kwiatkowski (5). The movements of the lever recording the contraction of the diaphragm slip were detected by a capacity-operated electronic relay; the relay then controlled the event timer. The system worked in such a way that a contraction of the diaphragm slip

caused the event timer to begin to record a vertical line on the kymograph paper: the next contraction stopped the stylus and it fell to the base line; the third contraction started the stylus upward again, the fourth stopped and returned it again, and so on. The resulting record was a series of vertical lines, proportional in height to the time elapsing between two breaths of the rat: a slowing of respiration therefore resulted in increased excursions of the stylus, and vice versa.

During the low-pressure phase of the cycle of artificial ventilation of the lungs, the rate of respiration was nearly constant, but in the high-pressure phase, when the intrapulmonary pressure suddenly rose to 10 cm of water pressure above atmospheric, there was an inhibition of respiratory rhythm which was recorded as a higher excursion of the stylus. This, of course, was caused by the operation of the Hering-Breuer reflex, the sensory arm of which originates in stretch receptors in the lungs. These receptors, when stretched by inflation of the lung, send nerve impulses by way of the vagus nerve, to the respiratory center, which have the effect of slowing the rhythmic rate of the respiratory pacemaker. This method of recording therefore automatically tests the working of the Hering-Breuer reflex at short intervals: the reflex responses appear on the record of the respiratory rate as higher excursions of

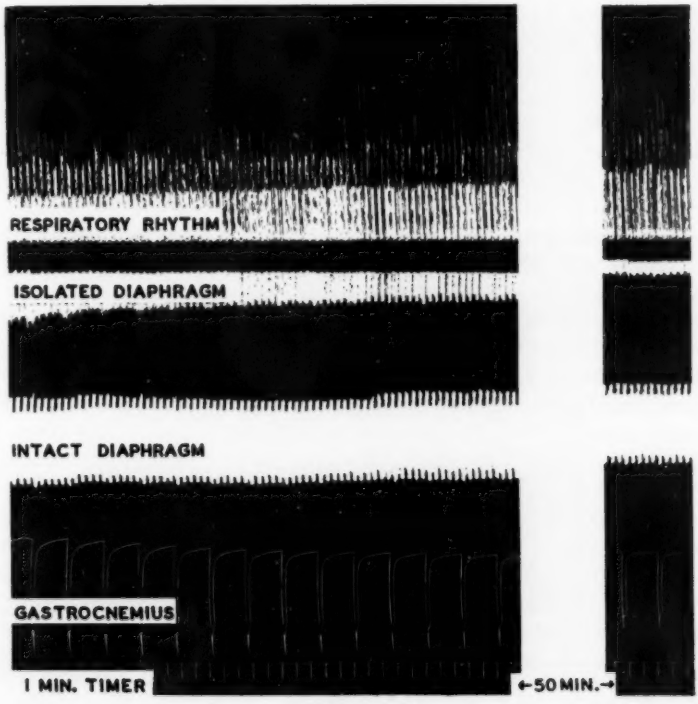

FIG. 1.

the stylus. At the slow paper speed used, these periods of respiratory inhibition are recorded as a ragged fringe of taller lines showing above the more constant and smaller excursions recorded during the deflation of the lungs. This is illustrated in the upper trace of Fig. 1.

Results

The Cause of Respiratory Arrest in Sarin Poisoning in the Rat, and the Site of Action of Atropine

In the first series of experiments, sarin was given intravenously, followed after a variable time by atropine. The doses of sarin used were 12, 25, 50, 100, and 200 μg per kg: duplicate experiments were done at each dosage. There was little variation in the results between duplicate experiments, and the effects of sarin increased proportionately as the dosage was raised. Figure 2 shows records obtained in three such experiments. A dose of 12 μg per kg of sarin had no effect on any of the recorded phenomena; 25 μg per kg caused rapid and complete respiratory arrest (as shown in the "intact diaphragm" trace*). After about two minutes there was a brief and partial recovery and then respiration ceased for about 15 minutes, at which time atropine was injected.

The respiratory paralysis was obviously purely central in origin because the isolated diaphragm slip, which had received no sarin, ceased to contract exactly as did the intact circulated right half of the diaphragm, whose neuromuscular junctions had been exposed to the sarin in the blood stream. Furthermore, the contractions of the gastrocnemius in response to stimulation of the sciatic nerve were not diminished in any way, showing that this dose of sarin did not cause generalized neuromuscular block. It may be concluded that this low dosage of sarin interferes with the function of the respiratory center but not with neuromuscular conduction. This result agrees with that found by deCandole *et al.* (2) for sarin in other species.

The Hering-Breuer reflex responses are shown not only in the record of respiratory rhythm but also in both diaphragmatic traces. In this particular rat, respiration ceased completely during the high pressure phase of artificial respiration. When the respiration was arrested by sarin, the reflex response of course was abolished. However, during the brief recovery of respiration after the initial arrest, the reflex was as active as before the sarin was given.

When a high dose of atropine (10 mg/kg) was injected intravenously into a rat whose respiration had been arrested by sarin, the respiratory center immediately recovered its normal function and began to send impulses down the phrenic nerves to the diaphragm. This was demonstrated in all three experiments shown in Fig. 2 by the simultaneous recovery of respiratory movements in both isolated and intact diaphragm muscles. In addition, the reflex modification of the respiratory rhythm by inflation of the lungs (Hering-

*The "intact diaphragm" traces show small regular movements of the diaphragm even when respiration is completely arrested. This is caused by the artificial ventilation inflating and deflating the lungs and so moving the diaphragm. These movements, of course, are not visible in the "isolated diaphragm" traces.

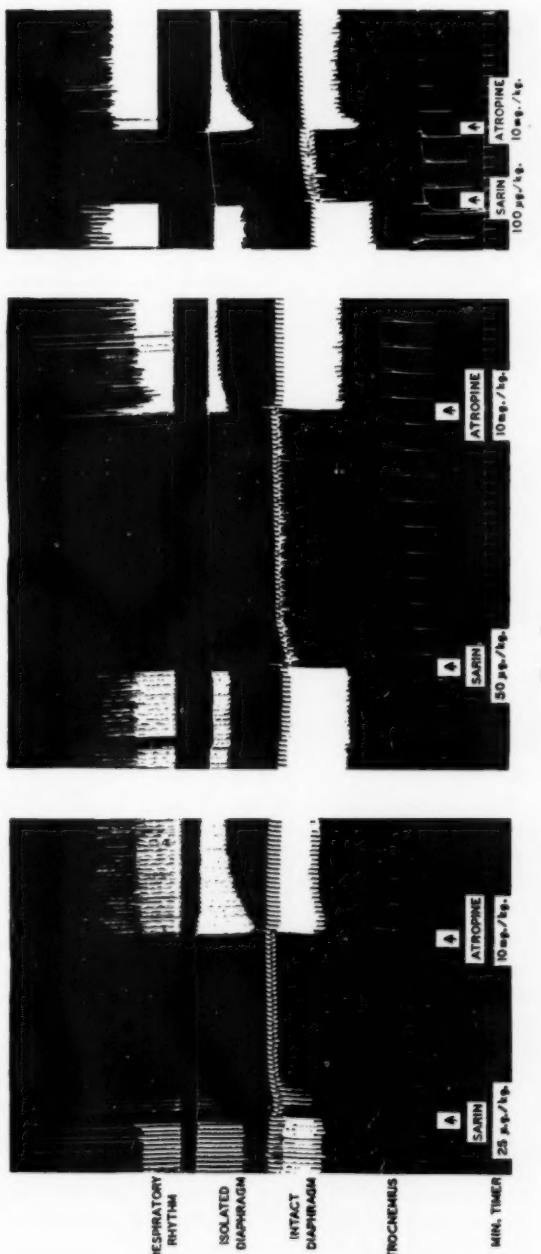
RESPIRATORY RHYTHM
ISOLATED DIAPHRAGM
INTACT DIAPHRAGM
GASTROCNEMIUS
1 MIN. TIMER
SARIN
25 µg./kg.
ATROPINE
10 mg./kg.
SARIN
50 µg./kg.
ATROPINE
10 mg./kg.
SARIN
100 µg./kg.
ATROPINE
10 mg./kg.

FIG. 2.

Breuer reflex) was also restored although the activity of the reflex was somewhat reduced. These effects appear to be due to an anticholinergic action of atropine at synapses in the respiratory center, or in other parts of the central nervous system concerned with the regulation of respiration.

Although atropine restored respiratory movements in both isolated and intact diaphragm muscles, it had no beneficial effect on neuromuscular conduction in the gastrocnemius: on the contrary, from the records, atropine itself had a slight neuromuscular blocking effect when given alone, and this appeared considerably greater when atropine was given after partial neuromuscular block by sarin. In the experiment with the highest dose of sarin, the respiratory movements of the intact diaphragm after restoration by atropine were definitely smaller than before the sarin was given; this indicates that partial neuromuscular blockade existed at the end plates of the circulated diaphragm after 100 μg/kg of sarin and 10 mg/kg of atropine.

It would be expected that still higher doses of sarin might cause complete neuromuscular blockade at the diaphragm. However, it was not possible to demonstrate such a condition because higher doses of sarin caused the rats to die of circulatory collapse.

In the isolated diaphragm slip, immediately after the administration of atropine, the recorded contractions attained a greater amplitude than in the period before administration of sarin, and then within a short time the contractions became smaller again: this increase in contractility may be attributable to the inactivity of the muscle during the respiratory arrest, which would allow the energy stored in the muscles to accumulate: from this it may be inferred that the oxygenation of the isolated preparation was not quite adequate to maintain the full effort of the muscle when driven by the respiratory center.

The Cause of Respiratory Arrest by Sarin, after Atropine

Larger doses of sarin could be given if atropine were injected first. Accordingly a second series of experiments was completed in which doses of sarin of 100, 200, 400, or 800 μg/kg were injected intravenously 10 minutes after 10 mg/kg of atropine. Two examples of the kymograph records obtained are reproduced in Fig. 3.

The injection of atropine (10 mg/kg i.v.) had no effect on the amplitude of the respiratory movements, or on the rate of respiration. However, the atropine partially depressed the activity of the Hering-Breuer reflex. There was also a slight impairment of neuromuscular conduction in the gastrocnemius, shown by a diminution in the tetanic contractions.

When sarin (100 or 200 μg/kg) was injected about 10 minutes after the atropine, it did not cause any depression of the respiratory center; there was only a slight brief diminution in the amplitude of the contractions of the diaphragm slip, accompanied by a transient increase in respiratory rate. This passed off in about one minute and the activity of the respiratory center, as registered by the isolated diaphragm slip, continued just as before the injection of the sarin. In contrast, the respiratory movements registered by the circulated diaphragm were gradually inhibited over a period of about 10 minutes

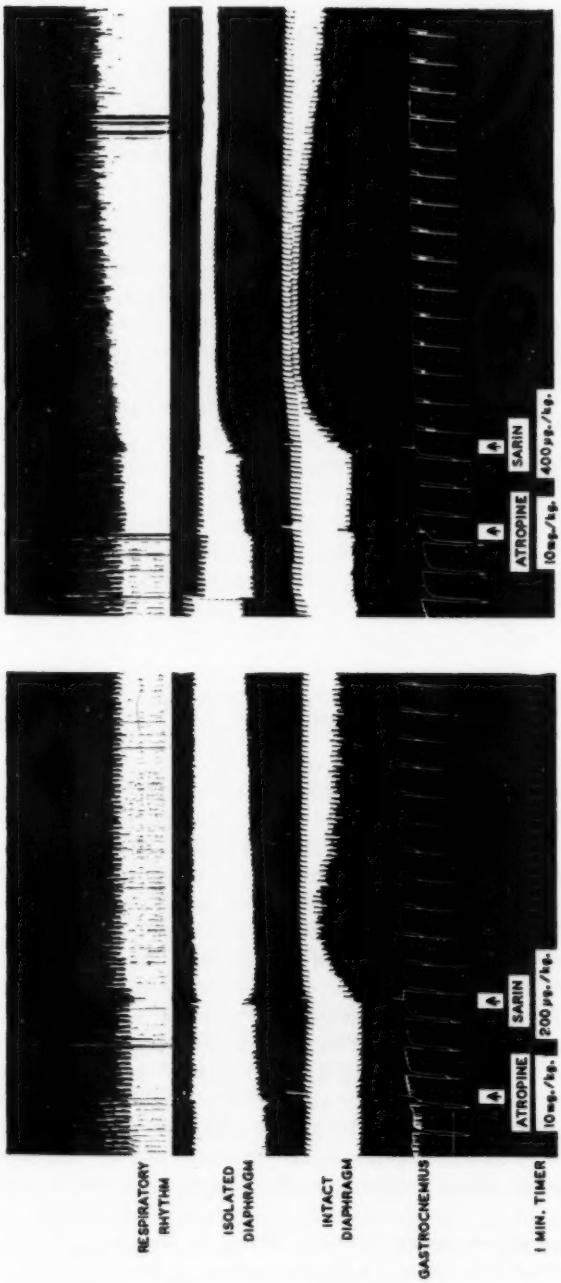
RESPIRATORY RHYTHM
ISOLATED DIAPHRAGM
INTACT DIAPHRAGM
GASTROCNEMIUS
1 MIN. TIMER
ATROPINE
10 μg./kg.
SARIN
200 μg./kg.
ATROPINE
10 μg./kg.
SARIN
400 μg./kg.

FIG. 3.

after injection of sarin, until the movements were reduced in amplitude to about one quarter of those recorded before the sarin was injected. This interference with respiration appears to have been due to neuromuscular blockade at the diaphragm. This interpretation is supported by the observation of simultaneous neuromuscular block in the gastrocnemius.

The peripheral respiratory paralysis, which gradually deepened for about 10 minutes after injection of sarin, then began to pass off. The partial recovery may have been due to a natural reactivation of inhibited cholinesterase at the neuromuscular junctions; or to adaptation of the junctions to comparatively large concentrations of acetylcholine, or possibly to other factors.

Whereas the respiratory inhibition, caused by 100 or 200 μg per kg of sarin after 10 mg/kg of atropine, was purely peripheral, when the dose of sarin was increased still further, central respiratory inhibition could again be demonstrated. Thus 400 or 800 μg/kg of sarin caused immediate diminution of the amplitude of the movements of the isolated diaphragm slip as shown in the second experiment of Fig. 3. When injected after atropine sarin obviously interferes with respiration by its effects both at the respiratory center and at the neuromuscular junctions of the diaphragm; the neuromuscular junctions recovered from the effects of the sarin, at least partially, within half an hour, but no signs of recovery of the respiratory center were observed.

In these experiments, injection of sarin caused a diminution of the activity of Hering-Breuer reflex, in addition to that already caused by the atropine. There was also a slight slowing of the respiratory rate after the higher doses of sarin.

Discussion

The Effect of Sarin on Respiratory Control

A small dose of sarin (e.g. 25 μg/kg) intravenously causes very rapid and complete paralysis of the respiratory center. This paralysis is relieved rapidly and completely by intravenous injection of a large dose of atropine. If the atropine is injected first, then larger doses of sarin are required to produce central respiratory depression and the depression becomes gradual instead of abrupt. When sarin was given without atropine, increasing the dose of sarin caused a very rapid increase in the effect on the respiratory center: for example, 12 μg/kg of sarin had no effect on the function of the respiratory center but 25 μg/kg caused immediate complete respiratory arrest. The relationship between dose and effect is quite different in rats given atropine: for example, after atropine, 200 μg/kg had no effect on the respiratory center, 400 μg/kg depressed the respiratory center about 50%, but 800 μg/kg had no greater effect than 400 μg/kg. In other words, the dose–response curve for the effect of sarin on the respiratory center is changed by atropine; the curve becomes displaced toward higher doses of sarin and its slope becomes much less steep.

When the respiratory center is first protected by atropine, and then depressed by sarin, although the output of nerve impulses to the diaphragm is reduced,

the rhythm of the respiratory efforts is only slightly slowed. From this it appears that the rhythm of the respiratory pacemaker is little affected by the sarin but that the number of outgoing pathways from the pacemaker to the respiratory muscles is reduced.

The Effect of Sarin on Neuromuscular Conduction

Small doses of sarin (25 μg/kg) caused respiratory arrest at the respiratory center but had no effect on neuromuscular conduction. With larger doses of sarin (100 μg/kg), there was severe neuromuscular blockade (as observed at the gastrocnemius) but this did not contribute to the respiratory paralysis which was already total because the respiratory center was completely inactivated. When the respiratory center was restored by injection of atropine, respiratory movements at the diaphragm were also restored almost completely.

In this case there is an apparent discrepancy between the degrees of neuromuscular block observed in the diaphragm as compared with the gastrocnemius: however, this difference may not necessarily represent a true difference in the degree of block present in the two muscles, because the nerve impulses arriving from the respiratory center may have been sufficiently desynchronized that a fairly efficient diaphragmatic contraction might result in spite of considerable muscular blockade. In other words, the comparison may be invalid because of differences in the type of stimulation supplied to the two muscles.

When atropine was given before the sarin, larger doses of sarin were required to depress the respiration. At 200 μg/kg of sarin, after atropine, there was no effect of sarin on the respiratory center but the respiratory output at the diaphragm was gradually depressed. This was clearly due to neuromuscular blockade at the diaphragm. Under these conditions the respiratory depression was purely peripheral.

When the dose of sarin was increased (400–800 μg/kg) the respiratory center was also depressed, so that the paralysis of respiration was partly peripheral and partly central. As time went by, there was partial recovery from the peripheral neuromuscular blockade, but not from the central paralysis.

The Effect of Sarin on Respiration as a Whole

In conclusion, the effect of sarin on the respiration of the rat may be summarized as follows: with low doses of sarin, respiration is suddenly arrested because of paralysis of the respiratory center; this effect is counteracted by high doses of atropine. After atropine, low doses of sarin have no effect on respiration; higher doses cause respiratory depression by neuromuscular blockade of the respiratory muscles.

Bronchoconstriction has been incriminated as a contributing cause to the respiratory difficulties in sarin poisoning (2, 3, 4) and in TEPP poisoning (1). In the present experiment, none of the respiratory records would be affected by bronchoconstriction because they were taken directly from the respiratory muscles; these experiments therefore offer no evidence concerning the part played by bronchoconstriction in sarin poisoning.

Acknowledgment

Material in this paper is published by permission of Defence Research Board of Canada.

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ACCLIMATIZATION TO COLD: IMMEDIATE ADRENAL RESPONSE AND SURVIVAL OF ACCLIMATIZED RATS EXPOSED TO MORE SEVERE COLD[1]

ROSEMARY MOLLOY, DORIS NICHOLLS, WILLIAM FARRINGTON, AND R. J. ROSSITER

Abstract

Further observations are described on the measurement of the incorporation of inorganic phosphate labelled with P^{32} into the inorganic phosphate of the adrenal gland to assess the immediate pituitary–adrenal response when cold acclimatized and non-acclimatized rats are exposed to more severe cold (2 hours at −5° C). In rats acclimatized to cold by conditioning to 3° C for 4 weeks, this immediate pituitary–adrenal response was considerably less than that in non-acclimatized rats maintained at room temperature (22° C). The reduction in the immediate pituitary–adrenal response took 3 to 4 weeks to develop and persisted for 12 hours, but not for 4 days. Rats that were conditioned to −5° C by exposures for 2 or 6 hours daily for 4 weeks showed no reduction in the immediate pituitary–adrenal response to more severe cold, but there was a significant decrease in this response in rats conditioned for 6 hours daily for 8 weeks.

Rats acclimatized to cold by conditioning to 3° C for 4 weeks showed greater survival when exposed to an environmental temperature of −15° C than rats conditioned to 22° C. Rats that were conditioned to −5° C for brief daily periods (2 hours or 6 hours) for 4 weeks or 8 weeks also survived exposure to severe cold (−22° C) better than rats maintained at room temperature.

In general, significant increases in adrenal weight were found in those cold-conditioned rats that showed a reduced pituitary–adrenal response. However, it is concluded that the development of increased survival on exposure to severe cold, by a process of conditioning to less severe cold, is not necessarily accompanied by a reduction in the immediate pituitary–adrenal response to severe cold, or by an increase in weight of the adrenal glands.

Introduction

Recently Nicholls and Rossiter (1) reported that the immediate adrenal response, as judged by the incorporation of inorganic P^{32} into the inorganic P of adrenal glands, was less in acclimatized rats conditioned to cold (3° C) than in non-acclimatized rats conditioned to a room temperature of 22° C, when both groups were subjected to more severe cold (−5° C) for a period of 2 hours.

Further observations are now reported on the development and persistence of this reduced immediate adrenal response in acclimatized animals. In addition, observations are reported on the survival rate when acclimatized and non-acclimatized rats were subjected to an environmental temperature of −22° C.

It is also shown that acclimatization can be produced by brief (2 hours or 6 hours) daily exposure of rats to an environmental temperature of −5° C.

[1]Manuscript received December 19, 1958.
Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario. The work was supported by grants from the Defence Research Board of Canada.

Methods

The incorporation of inorganic phosphorus labelled with P^{32} into the inorganic P of the adrenal gland and plasma of rats was measured. The animals were male rats of the Sprague–Dawley strain and weighed 200–300 g at the time of the experiment. They were allowed free access to food (Master Fox Cubes, Toronto Elevators Ltd.) and water. Two hours before being killed, each animal received 100 μc P^{32} as inorganic phosphate by intraperitoneal injection. The specific activity (counts/minute/μg P) of the adrenal inorganic P was expressed as a percentage of that of the plasma inorganic P (relative specific activity). Details of the analytical procedure have been described elsewhere (2).

Results

Immediate Adrenal Response to 2 Hours at −5° C

In the initial experiments, the results of which are shown in Table I, rats were maintained in individual cages at room temperature (22 ± 1° C) or in a

TABLE I

Persistence of reduced adrenal response

(Mean relative specific activity of adrenal inorganic P ± standard error of mean; number of animals in parentheses; *P* for comparison between the increase for the acclimatized rats and that for the non-acclimatized group)

Group	Controls (measured at 22° C)	Cold (2 hr at −5° C)	Increase	*P*	% increase
A Non-acclimatized (conditioned to 22 ± 1° C)	60 ± 3.5 (18)	124 ± 5.3 (26)	64 ± 5.3		107
B Acclimatized (conditioned to 3 ± 1° C)	73* ± 5.0 (14)	101 ± 6.3 (12)	28 ± 7.6	<0.01	38
C Acclimatized (conditioned to 3 ± 1° C + 12 hr at 22° C)	62 ± 5.3 (8)	86 ± 8.3 (8)	24 ± 9.8	<0.01	39
D Acclimatized (conditioned to 3 ± 1° C + 2 days at 22° C)	68 ± 4.0 (9)	120 ± 4.6 (6)	52 ± 5.9	>0.1	76
E Acclimatized (conditioned to 3 ± 1° C + 4 days at 22° C)	57 ± 3.0 (13)	128 ± 9.5 (9)	71 ± 9.2	>0.5	125

* Measured at 3° C.

cold room (3 ± 1° C) for a period of 4 weeks. These rats comprised groups A and B, respectively. At the end of this time, immediately after administration of P^{32}, one-half of the rats in each group was transferred to a second cold room maintained at −5° ± 1° C for the 2-hour period of the P^{32} incorporation. The remaining animals were left at the temperature to which they had been conditioned during the preceding 4 weeks.

In group A, exposure to −5° C for 2 hours caused a large (107%) increase in the incorporation of inorganic P^{32} into the inorganic P of the adrenal gland. In group B, exposure to −5° C for 2 hours caused a significant increase (38%) in the incorporation of inorganic P^{32}, but the increase was significantly ($P<0.01$) less than the increase observed in group A. This confirms the results of earlier experiments in this laboratory (1).

The mean increase for each group of rats was calculated from the difference between the mean relative specific activity of the cold-acclimated rats and that of the control rats. The probability that the mean increase of an acclimatized group differed significantly from the mean increase of the non-acclimatized group was calculated by the Student t test.

In order that the decrease in environmental temperature at the time of the experiment might be comparable for both acclimatized and non-acclimatized animals, rats that were conditioned to 3° C for 4 weeks (Table I) were returned to 22° C for 12 hours (group C), 2 days (group D), or 4 days (group E). At the end of this time, immediately after injection of P^{32}, one-half of each group was transferred to −5° C for the 2-hour period of the P^{32} incorporation, while the remaining animals were left at 22° C.

In group C there was a significant increase in the incorporation of inorganic P^{32}, which was of the same order (39%) as that observed in group B (38%), the acclimatized group that was not returned to 22° C for the 12 hours preceding the test. In group D the incorporation of inorganic P^{32} also was significantly increased. This increase in the immediate adrenal response (76%) was intermediate in value between that observed for group A, and that observed for group C. In group E the immediate adrenal response was significantly increased and was of the same order (125%) as that observed in group A (107%).

The incorporation of inorganic P^{32} into the adrenal gland of the control animals of group B, measured at 3° C, was significantly increased ($P<0.05$) over that of the control animals of group A, measured at 22° C. However, when the P^{32} uptake of the control animals of group C was measured at 22° C, the increase was not significantly different from that observed for the control rats of group A. This confirms earlier reports (1, 2).

Table II shows the results of experiments designed to measure the time required for acclimatization to develop. Rats were conditioned to 3° C for 1 (group F), 2 (group G), or 3 (group H) weeks before removal to 22° C for 12 hours, after which the experiments were carried out as already described. In all cases, the relative specific activities were significantly increased when the animals were exposed to more severe cold (−5° C) for 2 hours. However, the percentage increase in the immediate adrenal response was decreased as the period of conditioning increased from 1 to 4 weeks. The increases observed after 1 and 2 weeks (114%, 86%) were not significantly less than those observed in the non-acclimatized group A (107%). However, the increases observed after 3 and 4 weeks (57%, 39%) were significantly lower.

TABLE II

Time for reduced adrenal response to develop

(Mean relative specific activity of adrenal inorganic P ± standard error of mean; number of animals in parentheses; *P* for comparison between the increase for each group and the non-acclimatized group)

Group	Time at 3 ± 1° C (weeks)	Controls (measured at 22° C)	Cold (2 hr at −5° C)	Increase	*P*	% increase
A	0	60 ± 3.5 (18)	124 ± 5.3 (26)	64 ± 5.3		107
F	1	52 ± 3.5 (12)	111 ± 4.9 (13)	59 ± 6.0	>0.5	114
G	2	58 ± 5.1 (11)	108 ± 5.0 (14)	50 ± 8.0	>0.1	86
H	3	65 ± 6.8 (8)	102 ± 5.8 (12)	37 ± 8.7	<0.01	57
C	4	62 ± 5.3 (8)	86 ± 8.3 (8)	24 ± 9.8	<0.01	39

TABLE III

Development of reduced adrenal response by brief daily exposures to cold

(Mean relative specific activity of adrenal inorganic P ± standard error of mean; number of animals in parentheses; *P* for comparison between the increase for rats exposed to cold and those not so exposed)

Group	Conditioning procedure	Duration (weeks)	Controls (measured at 22° C)	Cold (2 hr at −5° C)	Increase	*P*	% increase
I	Not exposed to cold 22 hr at 22° C 2 hr at 25° C	4	56 ± 4.9 (7)	108 ± 5.7 (10)	52 ± 8.0		93
J	Exposed to cold 22 hr at 22° C 2 hr at −5° C	4	61 ± 2.4 (15)	120 ± 10.6 (16)	59 ± 11.2	>0.6	97
K	Not exposed to cold 18 hr at 22° C 6 hr at 25° C	4	49 ± 4.1 (14)	84 ± 6.6 (12)	35 ± 7.9		71
L	Exposed to cold 18 hr at 22° C 6 hr at −5° C	4	41 ± 1.3 (12)	76 ± 4.9 (13)	35 ± 5.3	>0.9	85
M	Not exposed to cold 18 hr at 22° C 6 hr at 25° C	8	48 ± 2.2 (10)	90 ± 3.1 (9)	42 ± 3.8		87
N	Exposed to cold 18 hr at 22° C 6 hr at −5° C	8	47 ± 3.2 (13)	72 ± 5.1 (14)	25 ± 6.1	<0.05	53

Table III shows the effect of brief daily exposures to cold on the immediate adrenal response. Rats kept at 22° C were transferred to a cold room maintained at −5 ± 1° C for 2 hours daily throughout a period of 4 weeks (group J). Other rats were transferred to a cold room maintained at −5° C for 6 hours daily throughout a period of either 4 weeks (group L) or 8 weeks (group N). Control rats kept at 22° C were transferred to a room maintained at 25 ± 3° C for comparable periods (groups I, K, and M respectively). In these experiments two rats were maintained in each cage during the con-

ditioning period. However, during the time of the P^{32} incorporation the rats were kept in individual cages.

Group J showed a significant increase in adrenal P^{32} incorporation when they were tested during an exposure for 2 hours at −5° C. The increase was not significantly different from that observed in rats of group I that had not been exposed to cold. Similarly, rats of group L that were conditioned to cold for 4 weeks by exposure for 6 hours daily showed an adrenal response that was not significantly different from that observed in the animals of group K that were not so exposed. However, rats of group N that were conditioned by exposure to −5° C for 6 hours daily for a period of 8 weeks did show a significant decrease in the immediate adrenal response when they were exposed to −5° C for 2 hours. This decrease in response (53%, compared with 87% for group M) was not as great as that found in group C after conditioning by continuous exposure to 3° C for 4 weeks (39%, compared with 107% for group A).

Adrenal Weight

Table IV shows that when rats were exposed to 3° C there was a statistically significant increase in the relative weight of the two adrenal glands at all time

TABLE IV

The effect of cold exposure on the weight of the adrenal gland

(Mean weight of the two adrenal glands per 100 g body weight ± standard error of mean; number of animals in parentheses; *P* for comparison between each group and the non-acclimatized group)

Group	Time at 3 ± 1° C (weeks)	Adrenal weight, mg/100 g body weight	% increase	*P*
A	0	17.4 ± 0.54 (53)		
F	1	22.2 ± 0.58 (21)	28	<0.001
G	2	22.8 ± 0.77 (22)	31	<0.001
H	3	27.7 ± 0.85 (22)	59	<0.001
C	4	25.0 ± 0.87 (21)	44	<0.001

intervals from 1 week to 4 weeks. An increase in adrenal weight following cold exposure was reported by Dugal and Therien (3), Woods (4), and Nicholls and Rossiter (2). At the end of 1 and 2 weeks, when the decrease in the immediate adrenal response was not statistically significant (Table II), the increase in the adrenal weight was moderate (28%, 31%). However, by 3 and 4 weeks, when the decrease in the adrenal response was greater, the increase in adrenal weight also was greater.

Table V shows that the enlargement of the gland was still present 4 days after the acclimatized animals had been transferred from the cold room to an environmental temperature of 22° C. By the fourth day at 22° C, the adrenal weight was significantly ($P<0.02$) less than that found in rats not removed

from the cold. It is of interest to note that by 4 days the effect of acclimatization on the adrenal response to cold was no longer present (Table I).

TABLE V

Changes in the adrenal weight of acclimatized animals

(Mean weight of the two adrenal glands per 100 g body weight ± standard error of mean; number of animals in parentheses; *P* for comparison between each group and the non-acclimatized group)

Group		Adrenal weight, mg/100 g body weight	% increase	*P*
A	Non-acclimatized (conditioned to 22 ± 1° C)	17.4 ± 0.54 (53)		
B	Acclimatized (conditioned to 3 ± 1° C)	25.3 ± 0.69 (27)	45	<0.001
C	Acclimatized (conditioned to 3 ± 1° C + 12 hours at 22° C)	25.0 ± 0.87 (21)	44	<0.001
D	Acclimatized (conditioned to 3 ± 1° C + 2 days at 22° C)	25.7 ± 1.04 (22)	48	<0.001
E	Acclimatized (conditioned to 3 ± 1° C + 4 days at 22° C)	22.9 ± 0.61 (25)	32	<0.001

TABLE VI

Changes in adrenal weight after brief daily exposures to cold

(Mean weight of the two adrenal glands per 100 g body weight ± standard error of mean; number of animals in parentheses; *P* for comparison between rats exposed to cold and those not so exposed)

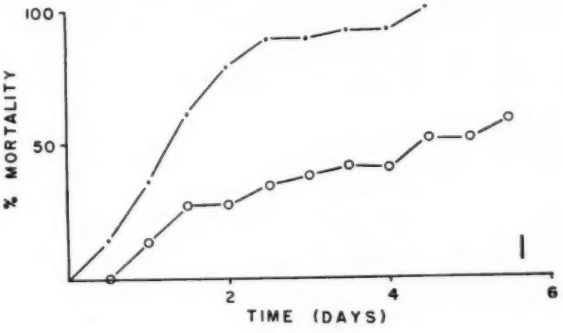
Group	Conditioning procedure	Duration (weeks)	Adrenal weight, mg/100 g body weight	% increase	*P*
I	Not exposed to cold 22 hr at 22° C 2 hr at 25° C	4	19.0 ± 0.53 (20)		
J	Exposed to cold 22 hr at 22° C 2 hr at −5° C	4	21.3 ± 0.63 (36)	12	<0.02
K	Not exposed to cold 18 hr at 22° C 6 hr at 25° C	4	18.2 ± 1.32 (26)		
L	Exposed to cold 18 hr at 22° C 6 hr at −5° C	4	19.2 ± 0.66 (25)	5	>0.4
M	Not exposed to cold 18 hr at 22° C 6 hr at 25° C	8	12.5 ± 0.32 (20)		
N	Exposed to cold 18 hr at 22° C 6 hr at −5° C	8	15.4 ± 0.34 (27)	23	<0.001

Table VI shows that there was very little increase in the relative weight of the adrenal glands of animals of group J or group L. There was a larger (23%) and statistically significant increase in the adrenal weight of rats of group N conditioned to cold by an exposure to −5° C for 6 hours daily throughout a period of 8 weeks. Although this increase was less than that

observed in rats of group C, after continuous exposure to 3° C for 4 weeks (44%), it was nevertheless accompanied by a reduced immediate adrenal response on exposure to more severe cold (Table III).

Acclimatization and Survival

Figure 1 shows the effect of exposure to severe cold (−15° C) on the mortality of non-acclimatized animals conditioned to 22° C for 4 weeks and acclimatized rats conditioned to 3° C for the same period. The rats had free access to food; water was given once a day. In the non-acclimatized group maintained at 22° C the 50% mortality time at −15° C was 30 hours. On the other hand, animals conditioned to 3° C had a 50% mortality time of 106 hours. By 5 days one-half of the acclimatized animals were still alive, whereas all of the non-acclimatized animals had died.



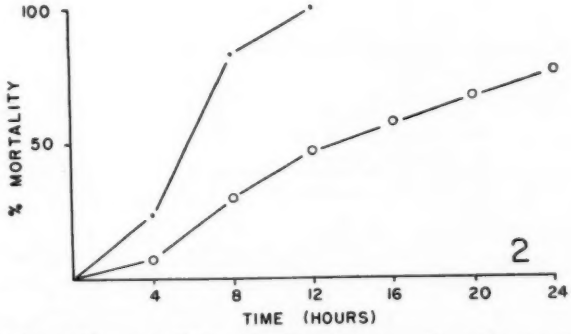
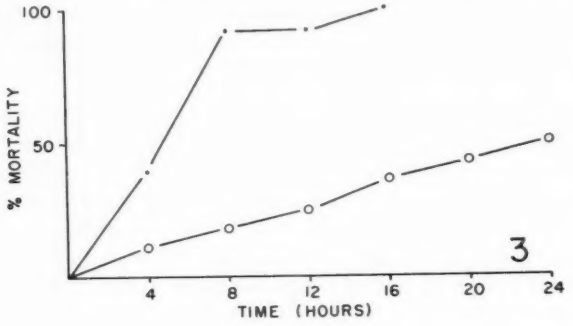


FIG. 1. Rats conditioned to 22° C or 3° C for 4 weeks. Mortality at −15° C.
•—• Not exposed to cold, i.e. conditioned to 22° C (36 rats).
○—○ Exposed to cold, i.e. conditioned to 3° C (30 rats).

FIG. 2. Rats conditioned to 25° C or −5° C for 2 hours daily for 4 weeks. Mortality at −22° C.
•—• Not exposed to cold, i.e. conditioned to 22° C for 22 hr and 25° C for 2 hr (30 rats).
○—○ Exposed to cold, i.e. conditioned to 22° C for 22 hr and −5° C for 2 hr (28 rats).



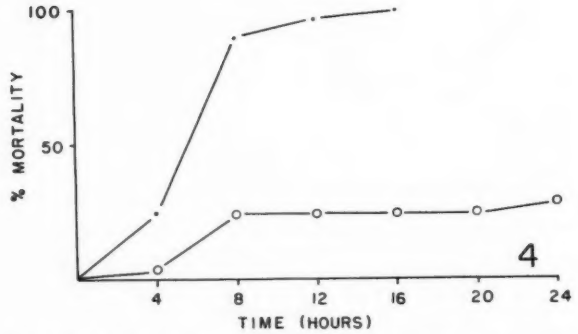


FIG. 3. Rats conditioned to 25° C or −5° C for 6 hours daily for 4 weeks. Mortality at −22° C.

•—• Not exposed to cold, i.e. conditioned to 22° C for 18 hr and 25° C for 6 hr (28 rats).

○—○ Exposed to cold, i.e. conditioned to 22° C for 18 hr and −5° C for 6 hr (28 rats).

FIG. 4. Rats conditioned to 25° C or −5° C for 6 hours daily for 8 weeks. Mortality at −22° C.

•—• Not exposed to cold, i.e. conditioned to 22° C for 18 hr and 25° C for 6 hr (30 rats).

○—○ Exposed to cold, i.e. conditioned to 22° C for 18 hr and −5° C for 6 hr (29 rats).

Figure 2 shows the effect of exposure to −22° C on the mortality of non-acclimatized animals maintained at 22° C and exposed to a temperature of 25° C for 2 hours daily and rats maintained under similar conditions but exposed to −5° C for 2 hours daily for 4 weeks. The cold-conditioned animals had a 50% mortality time of 13 hours compared to 6 hours for the animals not exposed to the cold environment. After 13 hours at −22° C all of the non-acclimatized rats had died, whereas one-half of the acclimatized animals were still alive.

Figures 3 and 4 show the effect of exposure to −22° C on the mortality of non-acclimatized animals maintained at 22° C and exposed to a temperature of 25° C for 6 hours daily and rats maintained under similar conditions but

exposed to −5° C for 6 hours daily for 4 and 8 weeks, respectively. For the animals not exposed to the cold environment the 50% mortality time was 5 hours and 6 hours, respectively, for the 4-week and 8-week groups. In the group exposed to −5° C for 6 hours daily for 4 weeks, the 50% mortality time was 24 hours; the animals conditioned in this manner for 8 weeks showed a considerably lower mortality. At the end of the 24-hour period the percentage mortality was only 28%. In both instances, when all of the non-acclimatized rats had died more than one-half of the acclimatized animals were still alive.

Discussion

Survival

It is well established that rats exposed to a cold environment for several weeks show an increased survival when exposed to more severe cold stress (5, 6). The results presented in Fig. 1 show that rats exposed continuously to 3° C for 4 weeks had a greatly increased survival when they were subsequently exposed to −15° C.

Of interest is the finding that when rats were exposed to a lower temperature (−5° C) for only short periods of time each day (Figs. 2 and 3) during a 4-week interval, there also was an increase in the survival when the animals were subsequently exposed to more severe cold (−22° C). The beneficial effects on survival were greater if the duration of exposure to the cold was 6 hours daily rather than 2 hours, and they were greater after 8 weeks than after 4 weeks of conditioning (Fig. 4). It should be noted that Heroux (7) reported an increase in the survival of rats conditioned to 6° C for several hours daily for 6 weeks.

Adrenal Response

The experiments show that rats that have been previously conditioned by continuous or brief daily exposure to cold are better able to survive exposure to more severe cold. Such acclimatization is associated with an increase in oxygen consumption, at least in rats conditioned by continuous exposure (8, 9, 10, 11, 12). The present results suggest that such an increased oxygen consumption is not the result of an increase in the activity of the pituitary–adrenal system. If the increased incorporation of inorganic P^{32} into the adrenal is considered an index of pituitary–adrenal activity, the immediate pituitary–adrenal response to further cold stress in animals conditioned continuously to cold is considerably less than that of non-acclimatized rats.

In rats conditioned by continuous exposure the time taken for the reduced adrenal response to develop was comparable to the time taken for similarly conditioned rats to show increased survival at 1.5° C after removal of the fur by clipping (6). Similarly, the reduced adrenal response disappeared rapidly when the animals were returned for 4 days to a warm environment, just as the increased survival at 1.5° C after clipping disappeared when the animals were returned to a warm environment following the period of continuous

conditioning. Fregly (13) found the time of onset of acclimatization was shorter (5–10 days), as judged by the rate of colonic cooling.

In the animals conditioned by continuous exposure to 3° C there was a greater increase in adrenal weight after 3 or 4 weeks than after 1 or 2 weeks, when the decrease in adrenal response became evident. Moreover, after the conditioned rats had been returned to a temperature of 22° C for 4 days and the reduced adrenal response had disappeared, there was a significant, though small, reduction in adrenal weight towards normal.

In rats conditioned by brief daily exposure (2 hours or 6 hours) to −5° C for a period of 4 weeks, there was no reduction in the immediate adrenal response to more severe cold, despite the observed increase in survival. Also these rats showed only small increases in adrenal weight. On the other hand, after conditioning by daily exposure to −5° C for 6 hours over a period of 8 weeks, there was a significant reduction in the immediate adrenal response to more severe cold, as well as an increase in adrenal weight.

It is concluded that the development of increased survival in the cold by a process of conditioning to less severe cold is not necessarily accompanied by a reduction in the immediate pituitary–adrenal response to more severe cold, or by an increase in adrenal weight. However, there appears to be some degree of association between the increase in adrenal weight and the decrease in the immediate pituitary–adrenal response. Some observations on the mechanism of the immediate adrenal response are presented in the following paper (14).

Acknowledgments

The authors are grateful to Mrs. Carolyn Graham and Mr. Harry Lovegrove for technical assistance with this investigation. Valuable suggestions made by Dr. J. A. F. Stevenson during the preparation of the manuscript are gratefully acknowledged.

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ACCLIMATIZATION TO COLD: OBSERVATIONS ON THE MECHANISM OF THE REDUCED IMMEDIATE ADRENAL RESPONSE[1]

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Abstract

The incorporation of inorganic phosphorus labelled with P^{32} into the inorganic phosphorus of the adrenal gland was measured in rats acclimatized to cold for 4 weeks. Previously it was reported that the immediate pituitary–adrenal response to brief cold exposure (2 hours at −5° C), as judged by the increased P^{32} incorporation is considerably decreased in rats that have been acclimatized. Some observations are now reported on the mechanism of this reduced immediate response.

The administration of ACTH, pitressin, or adrenaline caused similar increases in the adrenal phosphorus metabolism in acclimatized and non-acclimatized control rats. Acclimatization could still be demonstrated in rats after the fur had been removed by clipping.

From these results it is concluded that the decreased immediate pituitary–adrenal response to an exposure to more severe cold, observed in acclimatized rats, is not the result of an increased fur thickness, nor is it the result of a decreased sensitivity of the adrenal tissue to ACTH, or to a decreased sensitivity of the pituitary or hypothalamus to a given stimulus. It is suggested that the reduction in the immediate pituitary–adrenal response to a more severe cold stress in acclimatized rats might be due to an alteration in the sensitivity of the peripheral nerve receptors, or in the mechanism, nervous or otherwise, whereby the pituitary is stimulated.

Introduction

It is known that survival of rats and other small laboratory animals in a cold environment is enhanced by previous exposure to cold (1). Work in this laboratory has shown that the immediate response of the adrenal gland in rats acclimatized to a cold environment (2–4° C) is less than that observed in non-acclimatized animals conditioned to a temperature of 22° C, when both groups are subjected to a temperature lower (−5° C) than that of acclimatization (2, 3, 4). The adrenal response was estimated by determining the increase in the incorporation of inorganic phosphate labelled with radioactive phosphorus (P^{32}) into the inorganic phosphorus of the adrenal gland. Evidence in favor of the assumption that the incorporation of inorganic P^{32} into the adrenal may be used as an index of the stimulation of the adrenal cortex by ACTH has been summarized (3).

In order to investigate further the mechanism of the decreased immediate adrenal response in rats acclimatized to cold, the adrenal response to exogenous ACTH and to stimuli causing the release of endogenous ACTH, i.e. pitressin or adrenaline, was determined in both acclimatized and non-acclimatized animals. In addition, the possible role of changes in fur thickness was assessed

[1]Manuscript received December 19, 1958.
Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario. The work was supported by grants from the Defence Research Board of Canada and the National Cancer Institute.

by comparing acclimatized and non-acclimatized rats that were clipped prior to exposure to cold (3° C).

Methods

General

Young male rats (60–100 g) of the Sprague–Dawley strain were maintained in individual cages at room temperature (22 ± 1° C) or in a cold room (3 ± 1° C) for a period of 4 weeks. During this time, they were allowed free access to food (Master Fox Cubes, Toronto Elevators, Ltd.) and water. On the evening before the experiment, the animals that had been maintained in the cold (3° C) were returned to the room maintained at room temperature (22° C). Animals that were to receive ACTH or cortisone were returned to this room 17 hours prior to P^{32} administration; those that were to receive adrenaline or pitressin, as well as those that were to be clipped, were returned to room temperature 12 hours before the injection of P^{32}. On the day of the experiment, 2 hours before being killed, each animal received 100 μc P^{32} as inorganic phosphate by intraperitoneal injection. All animals were maintained at 22° C during the uptake of P^{32}, except clipped rats exposed to cold (3° C) for 2 hours.

The incorporation of inorganic P^{32} into the inorganic phosphorus of the adrenal gland and plasma was determined. The specific activity (counts/minute/μg P) of the adrenal inorganic P was expressed as a percentage of that of the plasma inorganic P (relative specific activity). Details of the analytical procedure have been described elsewhere (5).

ACTH, Cortisone, Adrenaline, and Pitressin

ACTH (4.0 I.U. per 100 g body weight) was given by intraperitoneal injection 2 hours before the P^{32} injection, i.e. 4 hours before killing.

Cortisone (5.0 mg cortisone acetate per 100 g body weight) was administered by subcutaneous injection, 20 hours before the P^{32} injection.

Pitressin (0.2 I.U. per 100 g body weight) or L-adrenaline (0.025 mg per 100 g body weight) was given by intraperitoneal injection immediately before the P^{32} administration.

The ACTH, pitressin, and adrenaline were products of the Parke-Davis and Company, Limited; the cortisone was a product of Merck and Company, Limited.

Clipping

The evening before the experiment the rats were returned to the room maintained at 22° C. They were lightly anaesthetized with sodium pentobarbital and clipped with an electric shaver. Control rats were treated similarly. Twelve hours later all of the rats received the P^{32}. Immediately afterwards one half of the animals were transferred to the cold room (3° C) for 2 hours, while the other half were maintained at 22° C.

Results

ACTH and Cortisone

Table I shows the effect of ACTH on the relative specific activity of the inorganic P of the adrenal gland of non-acclimatized and acclimatized rats,

TABLE I

Effect of ACTH on the incorporation of inorganic P^{32} into the inorganic P of the adrenal gland of non-acclimatized and acclimatized rats, with or without cortisone

(Number of animals in parentheses; P^1 for comparison of rats receiving ACTH with those receiving saline; P^2 for comparison of increases in non-acclimatized and acclimatized rats)

	Mean relative specific activity ± S.E.				
	Saline	ACTH	Increase	P^1	% increase
Non-acclimatized (conditioned to 22° C)	67.8±3.3 (13)	87.5±6.2 (12)	19.7±6.9	<0.01	29
Acclimatized (conditioned to 3° C)	72.2±6.7 (11)	95.7±5.1 (18)	23.5±8.4	<0.01	33
P^2			>0.7		
Non-acclimatized + cortisone (conditioned to 22° C)	52.8±5.0 (21)	71.1±5.4 (17)	18.3±7.4	<0.02	41
Acclimatized + cortisone (conditioned to 3° C)	63.8±5.9 (17)	82.2±7.3 (17)	18.4±9.3	<0.05	29
P^2			>0.9		

with or without the prior administration of cortisone. In all four groups, 4 I.U. ACTH caused a significant increase in the incorporation of inorganic P^{32} into the adrenal gland. There were no significant differences between the increases for acclimatized rats and non-acclimatized rats. The administration of cortisone caused a significant ($P < 0.05$) decrease in the relative specific activity of the adrenal inorganic P of rats receiving either saline or ACTH. This was true for both the non-acclimatized and the acclimatized animals.

Acclimatization to cold thus did not significantly affect the adrenal response to ACTH. The administration of cortisone caused a decrease in the incorporation of inorganic P^{32} into the inorganic P of the adrenal gland.

Adrenaline and Pitressin

Table II shows the effect of adrenaline and pitressin on the incorporation of inorganic P^{32} into the inorganic phosphate of the adrenal gland of non-acclimatized rats and rats acclimatized to cold for 4 weeks. In the non-acclimatized rats pitressin and adrenaline caused statistically significant increases in the mean relative specific activity of the adrenal inorganic P. This confirms the observations of Nicholls and Graham (6).

In the acclimatized rats pitressin and adrenaline also caused significant increases in the relative specific activities of the adrenal inorganic P. For pitressin the increase was 45% in the non-acclimatized rats and 61% in the acclimatized animals, while for adrenaline the increase was 43% in the non-acclimatized rats and 37% in the acclimatized group. In neither instance was the increase in the acclimatized rats significantly different from that found in the non-acclimatized animals.

TABLE II

The effect of adrenaline and pitressin on the incorporation of inorganic P^{32} into the inorganic P of the adrenal gland of non-acclimatized and acclimatized rats

(Number of animals in parentheses; P^1 for comparison of saline and test groups; P^2 for comparison of increases in non-acclimatized and acclimatized rats)

	Mean relative specific activity ± S.E.		
	Non-acclimatized (conditioned to 22° C)	Acclimatized (conditioned to 3° C)	P^2
Saline	59.6±2.9 (16)	53.8±3.6 (18)	
Pitressin	86.4±3.4 (16)	86.4±5.1 (13)	
Increase	26.8±4.4	32.6±5.8	>0.4
P^1	<0.01	<0.01	
% increase	45	61	
Adrenaline	85.1±4.5 (15)	73.6±3.2 (17)	
Increase	25.5±5.2	19.8±6.5	>0.5
P^1	<0.01	<0.01	
% increase	43	37	

Removal of Fur

Table III shows the results of experiments in which the fur was removed from a series of rats by clipping, after which the animals were subjected to a

TABLE III

The effect of exposure to cold on the incorporation of inorganic P^{32} into the inorganic P of the adrenal gland of non-acclimatized and acclimatized rats that were clipped

(Number of animals in parentheses; P^1 for comparison of rats in cold at 3° C with those maintained at 22° C; P^2 for comparison of increases in non-acclimatized and acclimatized rats)

	Mean relative specific activity ± S.E.				
	Controls at 22° C	Cold 2 hr at 3° C	Increase	P^1	% increase
Non-acclimatized (conditioned to 22° C)	56±4.2 (5)	94±1.1 (9)	38±6.9	<0.01	68
Acclimatized (conditioned to 3° C)	64±3.6 (11)	80±6.4 (13)	16±7.5	>0.05	25
P^2			<0.05		

cold environment (3° C) for 2 hours. In non-acclimatized rats (conditioned to 22° C) there was a significant increase in the relative specific activity of the inorganic P of the adrenal gland. On the other hand, acclimatized rats (i.e. conditioned to 3° C) exhibited a much smaller response when they were clipped and then returned to 3° C for 2 hours, indicating that the effect of acclimatization on the immediate adrenal response can be observed in animals deprived of fur.

Discussion

The experiments show that ACTH causes an increase in the relative specific activity of the adrenal inorganic P of both non-acclimatized and acclimatized rats, either with or without prior cortisone treatment. The observation that ACTH can cause an increase in the adrenal phosphorus metabolism in rats not previously hypophysectomized is of interest. Previously an effect of ACTH on P^{32} incorporation had been demonstrated only in hypophysectomized animals (6, 7). Intact rats may be used in the Sayers ascorbic acid depletion procedure (8), but hypophysectomized rats are much more suitable. Hodges (9) suggested that normal rats may be used if they are given deoxycorticosterone acetate prior to the ACTH injection.

In the present experiments, cortisone pretreatment decreased adrenal phosphorus metabolism; this confirms previous observations (2, 6), but it is of interest to note that cortisone did not affect the response to exogenous ACTH. Previously it was found that cortisone did not change the adrenal phosphorus response to exogenous ACTH in hypophysectomized rats (6). The direction of the change in adrenal phosphorus metabolism after the administration of cortisone is similar to that observed after hypophysectomy and is believed to be the result of a suppression of the release of ACTH from the anterior pituitary (3).

The experiments also show that the administration of ACTH to rats previously acclimatized to cold caused a significant increase in adrenal phosphorus metabolism, but that this increase was similar to that observed in the non-acclimatized control rats. Acclimatization by itself produced no significant changes in adrenal phosphorus metabolism in these experiments where the incorporation of P^{32} was measured 17 hours after the rats had been returned to room temperature. However, the immediate adrenal response to brief exposure to more severe cold ($-5°$ C) is less in acclimatized rats for a period of at least two days after the rats have been returned to room temperature (3, 4). The present results with exogenous ACTH strongly suggest that the decreased immediate adrenal response to cold in acclimatized rats is not due to a decreased ability of the adrenal tissue to respond, but rather to a decreased release of ACTH. These results are consistent with the observation of Heroux and Hart (10) that the eosinophil response to injected ACTH is almost normal by the fifth week of exposure to 6° C.

The experiments reported in Table II indicate that the effects of adrenaline and pitressin are as great in rats acclimatized to cold as in non-acclimatized rats. The adrenal response to adrenaline, as judged by ascorbic acid depletion, is abolished in rats with suitably placed hypothalamic lesions (11), suggesting that adrenaline acts on the hypothalamus. Evidence for the view that commercial pitressin contains a substance that can stimulate the release of ACTH, even in the presence of hypothalamic lesions that abolish the effect of adrenaline, is summarized by Nicholls and Graham (6). Presumably the active substance acts directly on the pituitary, although a report of Royce and

Sayers (12) indicates that large quantities of pitressin may deplete adrenal ascorbic acid even in hypophysectomized rats. In this regard it is of interest that Nicholls and Graham (6) also reported a small but statistically significant effect on the phosphorus metabolism of the adrenal of hypophysectomized animals that had received smaller doses of pitressin.

The observation that adrenaline, which is thought to stimulate the hypothalamus, and pitressin, which is thought to stimulate the anterior pituitary directly, are as effective in eliciting the adrenal response in acclimatized as in non-acclimatized rats, suggests that there is no change in the sensitivity of the hypothalamus or of the anterior pituitary in the acclimatized animals. Thus the reduced pituitary–adrenal response to cold in acclimatized rats is not due to a decrease in the sensitivity of the hypothalamic–pituitary system to a given stimulus, but rather to a decrease in the stimulus.

Previously, it was found that the administration of cortisone decreases the pituitary–adrenal response to adrenaline and pitressin, as well as the response to cold (2, 6). Cold-acclimatization decreases the pituitary–adrenal response to cold, but not the response to adrenaline and pitressin. These findings suggest that it is unlikely that the decreased adrenal response to cold in acclimatized animals is the result of any great increase in circulating adrenal corticoids.

The present observations do not explain the mechanism of the decreased pituitary–adrenal response to cold in the acclimatized rats. However, they do suggest that the decreased response is not the result of a decrease in the sensitivity of the adrenal cortex, the pituitary, or hypothalamus, to a given stimulus. In addition, the decreased response is not the result of an increased fur thickness, since removal of the fur by clipping did not eliminate the response.

It is possible that the reduction in the immediate pituitary–adrenal response to a more severe cold stress in acclimatized rats might be due to an alteration in the sensitivity of the peripheral nerve receptors, or in the sensitivity of the mechanism, nervous or otherwise, by which the pituitary is stimulated. Such a conclusion may perhaps be correlated with the anatomical changes reported by Heroux and St. Pierre (13) in the skin of the ears of acclimatized rats. The presence of peripheral changes in acclimatized animals also would help to explain the local acclimatization to cold described by some workers (14, 15). It should be stressed, however, that any change in the peripheral afferent mechanism is not complete and may be extremely selective, since the same peripheral receptors are presumably responsible for the initiation and maintenance of the whole machinery of cold acclimatization.

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EVALUATION OF PROTEIN IN FOODS

1. A METHOD FOR THE DETERMINATION OF PROTEIN EFFICIENCY RATIOS[1]

D. G. CHAPMAN, RAUL CASTILLO,[2] AND J. A. CAMPBELL

Abstract

A bioassay procedure for the evaluation of the nutritional quality of the protein in foods is described. This method involves measurement of the protein efficiency ratio (grams gain per gram protein consumed) under standardized conditions. Male rats of the Wistar strain 20–23 days of age are fed ad libitum an otherwise adequate reference diet containing 10% protein supplied by a standard sample of casein. Foods to be assayed are added to the diet as the sole source of protein at the expense of the casein and corn starch to maintain a protein level of 10%. Protein efficiency ratios (P.E.R.'s) are calculated after 4 weeks and are adjusted to a constant value of 2.5 for casein. Although influenced by the age of rat and subject to certain inherent criticisms, determination of P.E.R. values was found to be a simpler method for evaluating protein quality than determination of net protein retention or net protein utilization and equally sensitive.

Introduction

Methods of assessing the nutritive value of proteins have been extensively reviewed in the excellent articles of Allison (1, 2). Four of the main procedures are based upon (i) nitrogen balance, (ii) growth, (iii) tissue regeneration, and (iv) amino acid composition of the protein.

Growth is perhaps the most extensively used procedure and is often expressed in terms of protein efficiency ratio (P.E.R.) i.e., the ratio of grams body weight gained in a specified period of time to the grams of protein consumed. Protein efficiency ratios appear to be related reasonably well to other methods of evaluating proteins. Block and Mitchell (3) found there was a good relation between P.E.R.'s and biological values and also between P.E.R.'s and chemical score based on amino acid composition. Bender (4) reported good correlation between P.E.R. and net protein utilization.

Since protein efficiency ratios are well correlated with other methods of evaluating protein quality and since they are probably more widely used than any other method, it was decided to use this method for evaluating protein quality. While many protein efficiency ratios are reported in the literature, the methods by which they have been determined were so varied that it is difficult to compare them directly. In addition few workers have used a standard or control protein in their studies. It is the purpose of this paper (i) to describe a method for determining the protein efficiency ratio of foods, (ii) to compare this technique with certain others used to assess the nutritive value of proteins in foods, and (iii) to report the protein efficiency ratios of a variety of foods determined by the suggested procedure.

[1]Manuscript received October 3, 1958.
Contribution from Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario.

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Method

It is most important that any procedure be carried out under standardized conditions of protein level, species of animal, age of animal, use of an appropriate standard, duration of assay, and method of feeding. Having these considerations in mind the following method is described.

Animals. — Use weanling male rats of a single strain (Wistar) 20–23 days of age, 10 for each diet.

Diets. — Use a basal diet of the following percentage composition on an air-dried basis: corn starch — 80, corn oil — 10, non-nutritive cellulose* — 5, salts, U.S.P. XIV — 4, and vitamin mixture — 1. Incorporate the protein food under test into the diet at the expense of casein and corn starch to give 10% protein (N × 6.25). The protein content of the final diet should be within the range 9.7 to 10.3% determined by analysis. Prepare a vitamin supplement containing in 1 g the following amounts of vitamins: vitamin A — 200 International Units, vitamin D — 100 I.U., vitamin E — 10 I.U., vitamin K (menadione) — 0.5 mg, thiamine — 0.5 mg, riboflavin — 1.0 mg, pyridoxine — 0.4 mg, pantothenic acid — 4.0 mg, niacin — 4.0 mg, choline — 200 mg, inositol — 25 mg, para-aminobenzoic acid — 10 mg, vitamin B_{12} — 2 μg, biotin — 0.02 mg, and folic acid — 0.2 mg. Add sufficient cellulose to make 1 g. The vitamin levels supplied by this mixture are considered to be adequate but may be increased if felt desirable. Supply the diet and water ad libitum.

Reference Standard. — Maintain a group of rats on a diet consisting of the basal ration with casein as the source of protein. For uniformity, this casein should be the Test Diet High Nitrogen Casein approved by the Animal Nutrition Research Council and prepared by the Sheffield Chemical Co., Norwich, N.Y.

Assay Period. — Use a 4-week period.

Cages. — Use individual cages provided with feeders which will reduce food spillage to a minimum.

Randomization. — Use a randomized block design in which blocks represent variations in initial weight. Randomize the rats in each block for diet and cage. If variation between litters is great, use design to permit removal of this variable.

Determinations. — Maintain a weekly record of food consumption and body weight. Calculate the protein efficiency ratio by dividing the gain in weight by the grams of protein consumed, for each food and for the Reference Standard Casein in the same assay. Assuming the latter has a constant P.E.R. of 2.5 when determined under these conditions, correct the P.E.R. of the test food by the fraction $\frac{2.5}{\text{determined P.E.R. of Reference Standard Casein}}$.

Carcass Examination. — Examine carcass and organs for gross pathology. Determine liver fat content.

*Alphacel brand obtained from Nutritional Biochemicals, Cleveland, Ohio, U.S.A.

Experimental and Results

Effect of the Age of Rat and Length of Time on Test upon the Protein Efficiency Ratio of Casein

Forty male weanling rats were given the standard reference diet containing 10% protein supplied as casein. Using a randomized block design, 10 were placed on test at 22 days of age, 10 at 29, 10 at 36, and 10 at 43 days of age. It may be seen in Fig. 1 that the younger the rat when placed on test, the

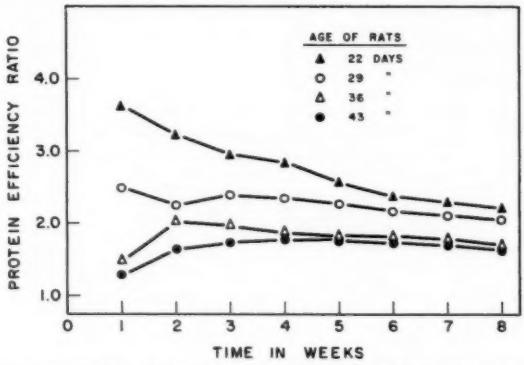


FIG. 1. Effect of initial age of rats on the P.E.R. of casein.

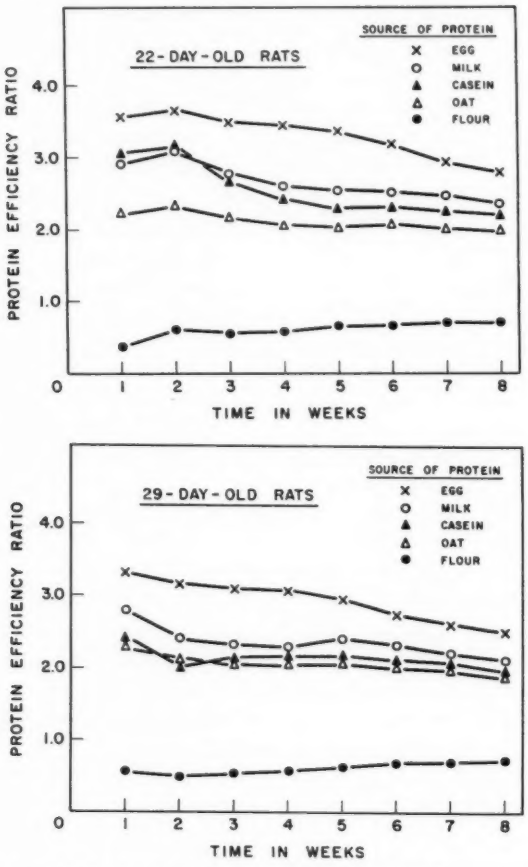
higher is the protein efficiency ratio obtained. This was true not only for the first few weeks, but throughout the entire 8 weeks of the test. At the fourth week on test, a rat placed on test at 29 days was found to have a P.E.R. approximately 20% less than that of a rat placed on test at 22 days of age. The decline in P.E.R. with length of time on test was most apparent in those on test at 22 days; however, in subsequent tests this decline was not as marked.

Effect of Age of Rat on P.E.R.'s of Egg, Milk, Wheat, and Oat Protein

Groups of rats 22 and 29 days of age were fed diets containing egg, milk, casein, oat, and wheat flour as the sources of protein. The results are shown in Figs. 2 and 3. As was found with casein, higher P.E.R.'s were obtained for egg, milk, and oat protein with 22-day-old rats than with 29-day-old rats. In the case of flour, there was little difference in the P.E.R.'s between those placed on test at 22 and 29 days. The diets containing egg, milk, and oat protein showed decreasing P.E.R. values as the length of time on test increased. It might be pointed out that at the 4-week period, using 22-day-old rats, the P.E.R. for rolled oats was significantly lower (at $P = 0.01$) than the P.E.R. of either milk or casein. However, using 29-day-old rats, there were no significant differences at the fourth-week period between oats and casein. This suggested that the 22-day-old rat was more sensitive to differences in protein quality than the 29-day-old rat.

Variation in Response of Rats

Coefficients of variation found in separate experiments for the P.E.R.'s and gains in weight are given in Table I. The data demonstrated that there was a



FIGS. 2 and 3. P.E.R. of egg, milk, casein, oats, and wheat flour using 22-day-old and 29-day-old rats respectively.

slight but useful gain in precision by calculating P.E.R.'s as compared to using simple weight gains. Coefficients of variation of P.E.R.'s tended to decrease from the first week and were usually lower at the third or fourth week, indicating that at this period the assay was more stable.

Comparison of Several Methods of Determining Protein Quality

Two new techniques of evaluating the nutritive value of proteins introduced by Bender and associates (4–6) were compared with the protein efficiency ratio method. The net protein ratio (N.P.R.) is obtained by adding together the loss in weight of a group of rats receiving a no-protein diet and the gain in weight of the test group and dividing by the grams of protein consumed by the test group. The net protein utilization (N.P.U.) is defined as the body nitrogen of the test group minus the body nitrogen of the no-protein group plus

TABLE I

Coefficients of variation in percentage for gains in weight and P.E.R.'s

		Gains in wt., week:				Protein efficiency ratios, week:			
	Expt.	1	2	3	4	1	2	3	4
Casein	1	12.9	10.8	15.8	18.4	15.1	16.9	9.4	9.5
	2	26.2	10.7	9.3	9.0	23.6	9.2	7.6	7.5
	3	23.3	17.2	8.2	11.4	16.5	17.5	2.7	8.6
	4	13.0	8.6	7.7	16.6	8.0	4.5	6.0	10.0
	5	17.8	10.2	8.4	11.3	12.5	7.4	5.5	6.6
	Mean	18.6	11.5	9.9	13.3	15.1	11.1	6.2	8.4
Egg powder	1	11.2	10.2	16.4	17.1	8.5	7.4	13.0	9.9
	2	13.4	8.5	9.4	3.1	17.2	8.7	4.1	3.7
	3	14.8	13.1	8.9	11.0	8.6	8.2	4.5	6.1
	4	16.5	10.6	8.4	7.2	12.9	6.8	8.4	4.5
	5	11.9	10.6	11.8	13.7	9.9	7.7	5.9	6.8
	Mean	13.6	10.6	11.0	10.4	11.4	7.8	7.2	6.2
Milk	1	11.3	10.0	14.2	12.3	8.0	6.8	8.4	6.2
Oatmeal	1	19.3	9.5	10.5	15.5	13.5	7.3	7.4	9.3
Flour, white	1	9.6	34.4	31.6	26.2	9.3	23.8	24.8	30.0

the nitrogen consumed by the no-protein group, the resulting value being divided by the nitrogen consumed by the test group. The N.P.R. and the N.P.U. were determined from a 1-week assay period, while the protein efficiency ratios were determined using the 4-week assay. Egg, milk, casein, rolled oats, and a "protein" cereal with and without milk were fed as the sources of protein in this study. The cereal and milk combination was fed in the ratio of 1 oz of cereal to 4 oz of whole fluid milk. The results of this study are shown in Table II. As would be expected, the N.P.R. values are greater than the

TABLE II

A comparison of three methods of expressing nutritive value of proteins (with standard error)

Food	P.E.R. ± S.E. 4-week assay	Net protein ratio ± S.E. 1-week assay	Net protein utilization ± S.E. 1-week assay
Egg	3.50±0.11	4.88±0.11	85.1±3.5
Milk	2.70±0.05	4.10±0.17	74.9±4.7
Casein	2.50±0.07	3.91±0.12	72.2±5.2
Oats, Quick	2.13±0.06	3.68±0.14	65.7±2.9
"Protein" cereal	0.03±0.02	1.61±0.17	33.2±3.3
"Protein" cereal plus milk	1.96±0.03	3.13±0.14	58.6±3.9

P.E.R. values. With the exception of the "protein" cereal, and egg, the variation within the 10 rats on each diet was greater for the N.P.R. than for the P.E.R. In this test also, the determination of the N.P.U. appeared to be more variable than did the determination of the P.E.R. While all three methods placed the foods in the same order, the P.E.R. values showed a greater spread between the poorest and the best protein source than did either of the other two techniques.

Protein Efficiency Ratios of Various Foods

In Table III the P.E.R. values are shown for a variety of foods determined by the assay procedure outlined above. Where the cereals were fed with milk,

TABLE III

The protein efficiency ratios of a variety of foods (with standard error)

Food	P.E.R. ± S.E.	Food	P.E.R. ± S.E.
Breads		Other foods	
White enriched	0.77 ± 0.03	Beans, lima, cooked	1.72 ± 0.04
Whole wheat	1.10 ± 0.01	Egg, whole, powdered	3.50 ± 0.11
"Protein" bread	1.29 ± 0.02	Fish flour	3.04 ± 0.06
Gluten	0.90 ± 0.03	Flour, white enriched	0.59 ± 0.05
		whole wheat	1.17 ± 0.03
Breakfast cereals		Gelatin	−1.25 ± 0.05
Bran cereal + milk	2.15 ± 0.05	Hamburger, raw, dried	2.68 ± 0.03
Corn flakes + milk	2.64 ± 0.06	Milk, whole, powdered	2.70 ± 0.05
"Protein" cereal	0.03 ± 0.02	Peas, cooked	1.57 ± 0.05
"Protein" cereal + milk	1.96 ± 0.03	Soybean flour	2.04 ± 0.03
Puffed oat cereal + milk	2.51 ± 0.03	Wheat germ	2.53 ± 0.05
Puffed rice + milk	2.45 ± 0.03		
Puffed wheat + milk	1.90 ± 0.04	Infant cereals	
Quick oats	2.13 ± 0.06	"Protein" cereal A	2.21 ± 0.05
Quick oats + milk	3.16 ± 0.06	"Protein" cereal B	2.42 ± 0.03
		"Protein" cereal C	2.13 ± 0.04

the ratio of 1 oz of cereal to 4 oz of whole fluid milk was used throughout. The milk was incorporated into the diet as whole milk powder. The level of 10% protein in the final diet was maintained. The P.E.R.'s of some breads available on the Canadian market were found to range from 0.77 to 1.29. The protein content of most of the ready-to-eat breakfast cereals is so low that when they are incorporated into the final diet at the maximum level of 80%, the level of protein is less than 10%. In view of this most of the cereals have been assayed only in combination with milk. While the P.E.R. value for "Quick Oats" is increased considerably when fed with milk, a much greater increase was observed in a so-called "Protein Cereal" where the P.E.R. rose from a value of 0.03 for the cereal alone to 1.96 when milk was added to the diet. The three infant cereals referred to in Table III are all mixtures of various cereals and contain from 28 to 34% protein.

Discussion

The determination of the protein efficiency ratio of a food as a measure of the nutritive value of its protein is subject to two major criticisms. Firstly, the method assumes that there is no protein requirement for maintenance; as larger quantities of protein are consumed, more is available for growth and a higher P.E.R. results. Secondly, the method assumes that the increase in body weight is proportional to the protein retained. This may not always be true since it has been reported that the composition of the weight increase varies with the type of diet (4). Nevertheless, in spite of these criticisms, the P.E.R. determination, because of its simplicity, has become the most

extensively used method of evaluating the nutritive value of the protein in foods.

The results of growth tests may be expressed either in terms of gain or gain per gram protein consumed, i.e. protein efficiency ratio. While Hegsted *et al.* (7) have questioned the need for the latter calculation, the Rutgers Collaborative Study (8) demonstrated very clearly that the variation between laboratories was reduced by approximately 50% by taking food consumption into account. The present study demonstrated a consistent increase in precision by this technique for in most cases lower coefficients of variation existed between P.E.R.'s than between corresponding weight gains. This result is to be expected since gains are related to feed intake.

Henry and Kon (9) have pointed out repeatedly the desirability of including some reference standard protein in all balance experiments since responses may vary markedly with different batches of rats. The present authors are convinced of the need for such a standard in P.E.R. determinations and have chosen casein as the reference protein. A large quantity of standardized casein has been set aside by the Animal Nutrition Research Council for this purpose.

Few authors who have reported P.E.R. values in the literature have stated the actual age of the rats when placed on test. The most commonly used term to describe the age is to state that "weanling" rats were used, or, to specify the weight of the rat. However, in view of the differences in initial weights of the rats mentioned in various papers, it seems probable that the term "weanling" has been used to describe rats which may vary considerably in age. The results reported here would suggest that the age of the rat when placed on test is quite critical with regard to the P.E.R. which may be obtained and that only rats between the ages of 20 and 23 days of age should be used if comparable results are to be expected between tests or laboratories. The effect of any differences in initial weight are eliminated by the design.

Bender (4) has pointed out that the P.E.R. determination is dependent upon food consumption and that food consumption varies considerably from one experiment to another. In the present studies, casein was assayed on five different occasions. The food consumption values per 100 g of body weight after 4 weeks on test amounted to 229, 238, 232, 237, and 229 g. The respective P.E.R. values for casein on these five tests were 2.41, 2.53, 2.55, 2.50, and 2.58. Bender has found the P.E.R. values for the dried skim milk to be 2.47, 2.68, 2.77, 1.70, 1.29, and 2.76 under his experimental conditions (4). It would appear, therefore, that if one is able to obtain uniform food consumption values through a standardized rat assay, that one of the major criticisms of the P.E.R. determination has been removed.

In the N.P.U. method as proposed by Bender (4) the determination of moisture, fat, and nitrogen on the rat carcass was tedious and time consuming and was not suitable for the routine determination of the nutritive value of the protein content of foods. It showed no significant improvement in results. It is concluded that the determination of P.E.R.'s under standardized condi-

tions as outlined offers a simple and reproducible procedure for evaluating protein quality. While there are certain criticisms inherent in the procedure it appears to be the most generally applicable method available at the present time.

There is a scarcity of information concerning the exact effect of level and type of protein on the fat content of the liver. In general, imbalance or inadequate amounts of amino acids tend to increase liver fat. While no criterion was proposed, it was felt that information on amount of liver fat might aid in the evaluation of protein quality in certain cases.

In a subsequent paper (10), the authors have made use of the P.E.R. values reported in this study in a proposed scheme for describing the protein value of foods.

Acknowledgment

The authors are indebted to Miss Constance E. Cox for advice on statistics and to Miss Margaret Jarvis and Mr. C. Perusse for the care and maintenance of the animals and for the chemical determinations.

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EFFECTS OF DIHYDROSTREPTOMYCIN ON AMINO ACID INCORPORATION INTO THE PROTEINS OF M. TUBERCULOSIS (BCG)[1]

E. STACHIEWICZ AND J. H. QUASTEL

Abstract

A study has been made of the effects of dihydrostreptomycin on amino acid incorporation into the proteins of *M. tuberculosis* (BCG). Suspensions of this organism on incubation at 37° with glycine-1-C^{14} give rise, aerobically, to labelled proteins in which 80% of the radioactivity appears in the glycine and serine moieties of the proteins and about 20% in alanine and aspartic acid. In presence of glycine-2-C^{14}, radioactivity appears in a larger number of amino acids of the protein. Incubation with serine-3-C^{14} leads to a distribution of radioactivity in the amino acids in BCG proteins but alanine-1-C^{14} and valine-1-C^{14} give rise to proteins with the radioactivity almost entirely in the corresponding amino acids. The process of aerobic incorporation of radioactivity from glycine-1-C^{14} in BCG proteins is stimulated by the presence of glucose, glycerol, sodium pyruvate, sodium stearate, or sodium benzoate in the medium in which the cells are incubated, the rate of incorporation being approximately constant over a period of 4 hours. The incorporation depends largely on the presence of oxygen. Dihydrostreptomycin (33 μg per ml) markedly inhibits labelling of proteins in the cell suspensions in presence of radioactive amino acids, the inhibition increasing with concentration of the streptomycin to an optimal concentration of 200 μg/ml. Penicillin and isonicotinic hydrazide are inactive but chloromycetin is an effective inhibitor. Cyanide, arsenite, and azide are inhibitory. The presence of lecithin stimulates incorporation of radioactivity from glycine-1-C^{14} into BCG proteins. Dihydrostreptomycin inhibitions of amino acid incorporation into BCG proteins increase with time of incubation of the cells with the drug. Concentrations of dihydrostreptomycin that inhibit labelled amino acid incorporation into labelled proteins by 50% have no effect on BCG respiration. The drug has no inhibitory effect on labelled amino acid incorporation in *E. coli* or Ehrlich ascites carcinoma cells in vitro but is effective with *M. phlei*. It does not affect selectively the distribution of radioactivities of the component amino acids of BCG proteins; only the total radioactivity incorporated into the proteins is diminished. The results lead to the conclusion that dihydrostreptomycin brings about an inhibition of protein synthesis in the BCG strain of *M. tuberculosis* at concentrations at which it exerts antibiotic effects.

Bernheim and Fitzgerald (1) have shown that streptomycin inhibits the oxidation of benzoic acid by *M. tuberculosis* and the formation of an adaptive enzyme attacking benzoic acid (2). Oginsky *et al.* (3) obtained a partial inhibition of the oxidation of higher fatty acids with this organism. Rosenblum and Bryson (4) observed that, with *E. coli*, the growth rate was inhibited by streptomycin only under favorable growth conditions. They suggested that streptomycin might exert its effect by inhibiting certain steps in nitrogen metabolism and protein synthesis rather than the oxidative processes of the cell as suggested by Oginsky *et al.* (5). Gale and Folkes (6) (see also 7, 8, 9) have shown that certain antibiotics such as aureomycin, terramycin, and chloramphenicol, in bactericidal concentrations, inhibit protein synthesis in *S. aureus* (Duncan) but they were unable to obtain a significant inhibition with bactericidal concentrations of streptomycin. Creaser (10) has, however,

[1]Manuscript received January 20, 1959.
Contribution from the McGill – Montreal General Hospital Research Institute, 3619 University Street, Montreal, Que.

demonstrated that streptomycin (100 μg/ml) greatly inhibits adaptive formation of β-galactosidase in resting cells of *Staphylococcus aureus*.

It was, therefore, of interest to determine whether streptomycin would inhibit amino acid incorporation into the proteins of tubercle bacilli. The following paper describes results obtained when *M. tuberculosis* (BCG) is exposed to dihydrostreptomycin in presence of radioactive amino acids and glucose.

Materials and Methods

M. tuberculosis, strain BCG, was grown on Dubos Tween-80 albumin medium (6) at 37° C. After 7 days the cells were harvested by centrifugation in the cold and washed twice with 0.05 *M* sodium phosphate buffer, pH 7.2, and suspended in this buffer solution.

A mixture containing 0.04 *M* phosphate buffer, 3.3 m*M* glucose, 4 m*M* amino acid, 100 μg of dihydrostreptomycin sulphate and 0.5 ml of a suspension of cells (10–14 mg dry weight of cells) was shaken in the standard Warburg manometric apparatus under aerobic conditions for 3 hours at 37° C. The volume of the mixture was 3 ml.

At the end of the experiment the reaction was stopped by adding 3 ml of 30% trichloroacetic acid. The cells were centrifuged, suspended in 6% trichloroacetic acid, and heated for 20 minutes at 90° C. They were then washed once with 6% trichloroacetic acid, once with 95% ethanol, suspended in alcohol: ether, and heated for 20 minutes at 50–60° C. The residue was washed once with ether and the extracted protein was suspended in acetone, plated, and counted.

The purified protein was hydrolyzed with 6 *N* HCl for 12 hours. The acid was removed by evaporating to dryness, dissolving in a few milliliters of water, and evaporating again. The residue was finally dissolved in 0.1 ml of water, which was then spotted on a sheet of Whatman No. 1 filter paper (10 in^2). Two-dimensional chromatograms were run in secondary butanol – formic acid – water (700 ml *sec*-butanol; 110 ml 90% formic acid; 170 ml water) in the first dimension and in phenol–water–ammonia (473 ml 90% phenol; 53 ml water; 1.7 ml ammonia sp. gr. 0.88) for the second dimension. The chromatograms were scanned to determine the amount of radioactivity in the spots. They were then placed next to X-ray films for a time depending on the amount of activity present (1-week exposure for a spot with 600 counts/minute).

Experiments were carried out to estimate the breakdown of glycine-1-C^{14} to $C^{14}O_2$ in presence of *M. tuberculosis* and to discover the effect, if any, of dihydrostreptomycin on this process. The conventional Warburg manometric apparatus was used in this work. A piece of filter paper (1 cm^2) was placed in the center well of the manometer vessels with 0.2 ml of 20% KOH. At the end of the incubation, 0.3 ml of 30% trichloroacetic acid was tipped from the side arm to stop the reaction and to liberate all the carbon dioxide from the medium. After 20 minutes the filter papers were removed and placed in 1 ml of 1.3% $NaHCO_3$ and the center well was washed out twice with water.

The filter paper was left in the solution overnight, then removed and washed. Two drops of *M* ammonium chloride solution and 0.5 ml of saturated barium chloride solution were added, mixed, and centrifuged. The precipitate was washed three times with water and once with acetone. It was finally suspended in 1 ml of acetone, and about one third of it plated and counted. The plates were weighed to determine the weight of the barium carbonate.

Results

Amino Acid Incorporation

Washed cells of *M. tuberculosis*, strain BCG, incubated in a phosphate medium, are capable of incorporating various amino acids into the bacterial protein. This process can be stimulated by the addition of certain oxidizable substrates such as glucose, glycerol, or pyruvate (11). Typical results are presented in Table I. The rate of incorporation of radioactivity from glycine-1-C^{14}, measured over a period of 4 hours, is linear (Fig. 1). Other radioactive amino acids such as L-alanine, L-leucine, and L-valine also bring about labelling of the protein. L-Glutamic acid, on the other hand, is apparently not utilized in this manner by the cells. Radioactivity of L-methionine is also incorporated at a feeble rate (Table II).

TABLE I

The effects of different substrates on the incorporation of radioactivity from glycine-1-C^{14} into BCG proteins

Substrate added	Incorporation, counts/min/mg protein
None	95
2 m*M* glycerol	164
3 m*M* glucose	222
3 m*M* sodium pyruvate	203
1 m*M* sodium stearate	113
3 m*M* sodium benzoate	127

NOTE: Vessel contents:
0.5 ml BCG cell suspension in 0.05 *M* sodium phosphate buffer solution,
1.5 ml of .05 *M* phosphate buffer,
0.2 ml of .06 *M* glycine-1-C^{14} (130,000 counts/min),
0.2 ml of substrate.
Water added to a final volume of 3 ml.
Incubation 3 hours at 37° C.

Inhibitors of Incorporation

The process of incorporation of radioactivity from labelled amino acids into the cells of *M. tuberculosis* BCG is sensitive to the presence of dihydrostreptomycin sulphate (Table II). Increasing concentrations of the drug produce an increasing inhibition (Fig. 2), but the increase is not directly proportional to the drug concentration. It appears that a maximal inhibition is reached at a dihydrostreptomycin concentration of about 200 μg/ml and that further increase of the antibiotic concentration gives only a small increase in the percentage inhibition. It seems that some incorporation can still take place at high dihydrostreptomycin concentrations.

Chloromycetin inhibits glycine-C^{14} incorporation but penicillin and isonicotinic acid hydrazide have little or no effect. Metabolic poisons such as cyanide, arsenite, and azide are inhibitory to different extents. Typical results are shown in Table III.

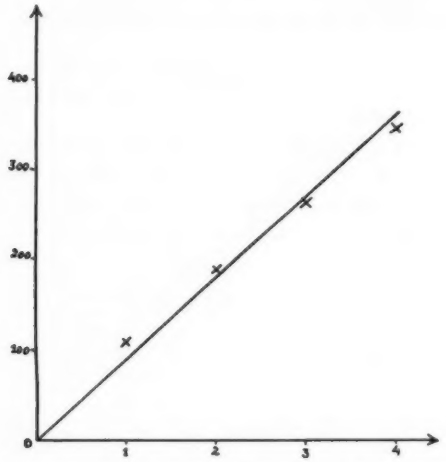

FIG. 1. Rate of incorporation of radioactivity from glycine-1-C^{14} into proteins of *M. tuberculosis* (BCG).
Ordinate: Incorporation, counts/minute/mg protein. *Abscissa:* Time, hours.
Experimental conditions: Vessels contained:
1.5 ml 0.05 *M* phosphate buffer pH 7.2,
0.2 ml 0.05 *M* glucose,
0.2 ml 0.06 *M* glycine-1-C^{14} (100,000 counts/minute),
0.5 ml suspension BCG cells in phosphate buffer.
Water to 3 ml. Aerobic, 37°.

TABLE II

The effects of dihydrostreptomycin on the incorporation of radioactivity from various amino acids into the proteins of *M. tuberculosis*, strain BCG

		Incorporation, counts/min/mg protein	
Amino acid added	Counts/min/vessel	Control	With dihydrostreptomycin
Glycine-1-C^{14}	110,000	150	90...(1)
Glycine-2-C^{14}	67,000	209	101...(1)
Alanine-1-C^{14}	288,000	446	237...(2)
Leucine-1-C^{14}	320,000	490	267...(2)
Valine-1-C^{14}	292,000	328	205...(2)
Glutamic acid-1-C^{14}	295,000	17	12...(2)
Methionine-S^{35}	167,000	61	48...(1)

NOTE: Vessels contained:
1.5 ml of 0.05 *M* phosphate buffer solution, pH 7.2,
0.2 ml of .05 *M* glucose,
0.2 ml of .06 *M* L-amino acid,
0.5 ml BCG cell suspension in phosphate buffer,
(1) 100 μg of dihydrostreptomycin sulphate in 0.1 ml,
(2) 300 μg of dihydrostreptomycin sulphate in 0.3 ml.
Water to 3 ml final volume.
Incubation 3 hours at 37° C.

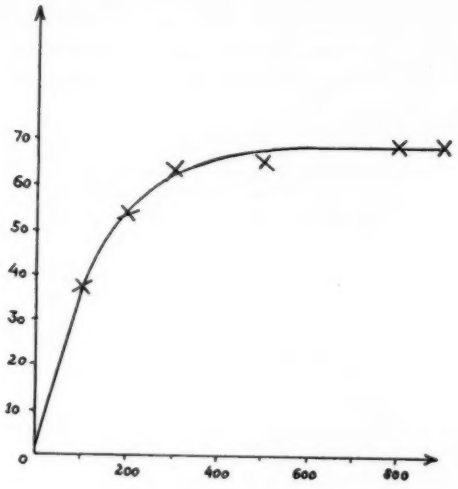

FIG. 2. Effects of variation of concentration of dihydrostreptomycin on incorporation of radioactivity from glycine-1-C^{14} into proteins of *M. tuberculosis* (BCG)
Ordinate: Percentage inhibition of incorporation.
Abscissa: Micrograms of dihydrostreptomycin sulphate.
Experimental conditions: Vessels contained:
1.0 ml 0.05 *M* phosphate buffer pH 7.2,
0.2 ml 0.05 *M* glucose,
0.2 ml 0.06 *M* glycine-1-C^{14} (100,000 counts/minute),
0.5 ml suspension of BCG cells in phosphate buffer,
dihydrostreptomycin sulphate made up in phosphate buffer.
Total vol. = 3 ml. Aerobic, 37°.

TABLE III

Effect of various inhibitors on the incorporation of radioactivity from glycine-1-C^{14} or glycine-2-C^{14} into the protein of BCG

	Incorporation, counts/min/mg protein
Expt. 1. Control (with glycine-1-C^{14})	126
" +100 μg of dihydrostreptomycin sulphate	69
" +5000 units of penicillin G	130
" +1 mg isonicotinic acid hydrazide	123
Expt. 2. Control (with glycine-2-C^{14})	305
" +5 m*M* sodium azide	198
Expt. 3. Control (with glycine-2-C^{14})	145
" +300 μg dihydrostreptomycin sulphate	67
" +300 μg chloromycetin	5
Expt. 4. Control (with glycine-1-C^{14})	365
" +7 m*M* sodium cyanide	22
" +3 m*M* sodium arsenite	182

NOTE: Vessels contained:
1.5 ml of 0.5 *M* phosphate buffer, pH 7.2,
0.2 ml of 0.5 *M* glucose,
0.2 ml of 0.6 *M* glycine-1-C^{14} (100,000 counts/min),
0.2 ml of 0.6 *M* glycine-2-C^{14} (67,000 counts/min),
0.5 ml BCG cells in phosphate buffer pH 7.2,
0.1–0.5 ml of inhibitor; water up to 3 ml.
Incubation 3 hours at 37° C.

The incorporation of radioactivity from glycine depends almost entirely on the presence of oxygen. The results given in Table IV show that under anaerobic conditions, even in the presence of glucose, the incorporation is almost completely inhibited.

TABLE IV

The incorporation of radioactivity from glycine-1-C^{14} under aerobic and anaerobic conditions

	Incorporation, counts/min/mg protein	
	Air	Nitrogen
Control	235	30
Control + 300 μg of dihydrostreptomycin	115	23

NOTE: Vessels contained:
1.5 ml of 0.05 *M* phosphate buffer solution,
0.2 ml of .05 *M* glucose,
0.2 ml of .06 *M* glycine-1-C^{14} (122,600 counts/min/vessel),
0.5 ml BCG cells in phosphate buffer,
0.3 ml of dihydrostreptomycin sulphate.
Water to final volume of 3 ml.
Anaerobic: Vessels gassed with pure N_2 for 10 minutes.
Incubation 3 hours at 37° C.

The Effect of Lecithin

Animal lecithin,* when added to the incubation medium, stimulates the incorporation of glycine-1-C^{14} radioactivity very markedly (Fig. 3). This effect is probably a physical one. Since the surface of mycobacteria consists of a fat layer, substrates probably do not enter the cells at a very rapid rate. Lecithin, being a surface active agent, may, therefore, render the cell more permeable to the substrate and thus increase its metabolic rate. The presence of lecithin produces a homogenous suspension of the tubercle bacilli cells, in contrast to the normal appearance of the cell suspension in which some of the cells form a surface film. Sodium tauroglycocholate, which is also a surface-active agent, has a similar effect on the cell suspension and also stimulates incorporation of radioactivity from glycine-1-C^{14} into the cell proteins.

Effect of Time of Incubation with Dihydrostreptomycin

Results shown in Table V indicate that the inhibitive effect of dihydrostreptomycin on amino acid incorporation into BCG protein is dependent on the

TABLE V

Effects of time of incubation of *M. tuberculosis* BCG cells with dihydrostreptomycin on the inhibition of incorporation of radioactivity from glycine-1-C^{14}

Time of preincubation with dihydrostreptomycin (hours at 37°)	% Inhibition of incorporation of radioactivity from glycine-1-C^{14} into BCG proteins
Nil	15
1	31
2	48

* Purchased from National Biochemicals Co., 90% pure.

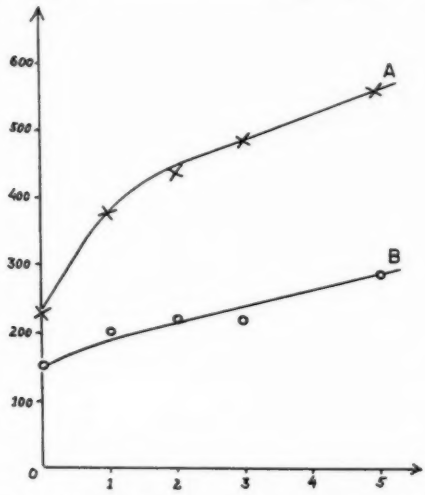

FIG. 3. Effects of lecithin on the incorporation of radioactivity from glycine-1-C^{14} into the proteins of *M. tuberculosis* (BCG).
Ordinate: Incorporation, counts/minute/mg protein.
Abscissa: Milligrams lecithin.
Curve A—No dihydrostreptomycin present.
Curve B—100 μg dihydrostreptomycin present.
Experimental conditions: Vessels contained:
1.5 ml 0.05 *M* phosphate buffer pH 7.2,
0.5 ml suspension of BCG cells in phosphate buffer,
0.2 ml 0.05 *M* glucose,
0.2 ml 0.06 *M* glycine-1-C^{14} (100,000 counts/minute),
0.1 to 0.5 ml lecithin solution containing
1 to 5 mg lecithin,
100 μg dihydrostreptomycin sulphate.
Water to 3 ml. Aerobic, 37°.
Incubation time 3 hours.

time of exposure of the cells to the antibiotic. These results would be consistent with the conclusion that the antibiotic penetrates slowly into the cells, where it presumably accomplishes its inhibitory effects.

The procedure in these experiments was to incubate 0.5 ml *M. tuberculosis* BCG cell suspension in 0.05 *M* phosphate buffer, 0.2 ml 0.05 *M* glucose, 0.1ml dihydrostreptomycin (100 μg), 1.5 ml 0.05 *M* phosphate buffer, and 0.5 ml water for various intervals of time in flasks shaken at 37° C in air. After these periods, 0.2 ml 0.06 *M* glycine-1-C^{14} (200,000 counts/minute) was added to the mixture and incubation carried out for 1 hour at 37° C. Radioactivity of the isolated proteins of the washed cells at the end of the experiment was estimated.

Respiration of M. tuberculosis (BCG)

The concentrations of dihydrostreptomycin which produce a 50% inhibition of amino acid incorporation have no effect on the respiration of the BCG cells (Fig. 4). Lecithin stimulates the rate of respiration as well as that of amino acid incorporation.

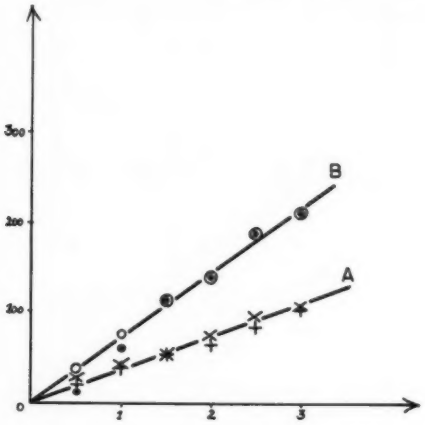

FIG. 4. Effects of dihydrostreptomycin on the respiration of *M. tuberculosis* (BCG).
Ordinate: Oxygen uptake, μl.
Abscissa: Time, hours.
Curve A, no lecithin present.
Curve B, 3 mg lecithin added.
○ and +, 100 μg dihydrostreptomycin added.
● and ×, no dihydrostreptomycin added.
Experimental conditions: Vessels contained:
1.5 ml 0.05 *M* phosphate buffer pH 7.2,
0.2 ml 0.05 *M* glucose,
0.2 ml 0.06 *M* glycine,
0.5 ml suspension BCG cells in phosphate buffer,
lecithin, dihydrostreptomycin sulphate.
Water to 3 ml. Aerobic, 37°.

Radioactive Amino Acids Present in the Labelled Cell Proteins

Hydrolyzates of the protein isolated from the BCG cells give spots on a chromatogram which correspond to the following amino acids: glutamic acid, alanine, leucine, arginine, valine, aspartic acid, isoleucine, lysine, serine, phenylalanine, tyrosine, histidine, threonine, methionine, and glycine.

If the cells are incubated with glycine-1-C^{14}, the hydrolyzate of the protein shows that 80% of the radioactivity appears in the glycine and serine and about 20% in alanine and aspartic acid. However, when glycine-2-C^{14} is used radioactivity appears in a large number of amino acids. Glycine and serine still contain the highest activity, but glutamic acid, aspartic acid, and alanine are also fairly active and there is some radioactivity in arginine, valine, and leucine.

Both alanine-1-C^{14} and valine-1-C^{14} give rise to proteins with the radioactivity remaining almost entirely in the corresponding amino acid. The only other amino acid in the protein showing very slight activity is glycine.

Leucine-1-C^{14} is incorporated mostly as leucine, but some of the C^{14} appears also in glycine and alanine and a very small amount can be found in aspartic and glutamic acids.

When the cells are incubated with serine-3-C^{14} the radioactivity is distributed throughout most of the amino acids. The greatest amount is found in

serine, glutamic acid, and alanine; next in activity are aspartic acid and valine. There is also some radioactivity in threonine, proline, arginine, lysine, and leucine. No radioactivity appears in the glycine spot.

These results lead to the conclusion that glycine is converted to serine in *M. tuberculosis* BCG, both amino acids being incorporated into the cell proteins. Decarboxylation of glycine also occurs, however, the methylene carbon finding its way into a variety of amino acids. A similar phenomenon occurs with serine. Thus, under the conditions of these experiments, protein synthesis in the tubercle bacilli takes place together, possibly, with amino acid exchange reactions (12).

The presence of dihydrostreptomycin seems not to affect in any selective manner the distribution of radioactivities of the component amino acids of the tubercle bacilli proteins. Only the total radioactivity incorporated into the protein is decreased.

Effect of Dihydrostreptomycin on the Incorporation of C^{14} from Glucose-U-C^{14} into the Proteins of M. tuberculosis (BCG)

Experiments were carried out to observe whether streptomycin would affect the incorporation of the label into cell proteins from radioactive glucose, when *M. tuberculosis* BCG was incubated in a glucose–phosphate medium containing no amino acid (13).

Results presented in Table VI show that the antibiotic (100 μg/3 ml) brings about a diminished rate of incorporation of C^{14} amounting to about 50%.

TABLE VI

The incorporation of radioactivity from glucose-U-C^{14} into the proteins of BCG

	Incorporation, counts/min/mg protein
Control	190
Control + 100 μg of dihydrostreptomycin	98

NOTE: Vessels contained:
2.0 ml of 0.05 *M* phosphate buffer,
0.2 ml of 0.05 *M* glucose-U-C^{14} (116,000 counts/min),
0.5 ml BCG cells in phosphate buffer.
Water to 3 ml final volume.
Incubation 3 hours at 37° C.

Such a result is consistent with the observation that amino acid incorporation is also inhibited, for doubtless the labelled glucose is converted in the cell into a variety of radioactive amino acids.

Discussion

The results that have been described show that there is a very active amino acid metabolism in *M. tuberculosis* BCG cells. Glycine is decarboxylated at a very rapid rate and most of the CO_2 comes from the carboxyl group (Table VII). The remainder of the carbon chain from both glycine and serine can be built into most of the other amino acids. These processes are not influenced by dihydrostreptomycin, which appears to inhibit only the step or steps by which the different amino acids are built into the protein molecule.

TABLE VII

The decarboxylation of glycine-1-C^{14} and glycine-2-C^{14} by tubercle bacilli BCG

	Counts/min/mg of $BaCO_3$	
	Control	Dihydrostreptomycin
Glycine-1-C^{14}	500	465
Glycine-2-C^{14}	77	65

NOTE: Vessels contained:
1.5 ml of 0.05 *M* phosphate buffer solution,
0.2 ml of .05 *M* glucose,
0.2 ml of .06 *M* glycine (31,000 counts/min),
0.5 ml of BCG cells in phosphate buffer,
0.3 ml dihydrostreptomycin (300 μg).
Water to 3 ml final volume.
Incubation 90 min at 37° C.

The inhibitory action of dihydrostreptomycin seems to be most marked in the mycobacteria. Gale and Folkes (6, 14) using *S. aureus* (Duncan) were unable to obtain any inhibition of amino acid incorporation with concentrations of dihydrostreptomycin similar to those described in this paper. We have also found that dihydrostreptomycin does not significantly inhibit amino acid incorporation in *E. coli* and Ehrlich ascites carcinoma cells in vitro. Using *M. phlei*, however, an inhibition was obtained similar to that found with *M. tuberculosis*.

The incorporation is dependent upon energy which is not available under anaerobic conditions even in the presence of glucose. Since dihydrostreptomycin has no effect on the respiration (Fig. 4), its inhibition of incorporation, is not the result of an over-all diminution of oxidative processes in the cell. Possibly its action is connected with a combination with nucleic acids (15). Much more work has to be done before a clear picture of the mode of action of this antibiotic can be obtained. The present results, however, lead to the conclusion that dihydrostreptomycin brings about inhibition of protein synthesis in the BCG strain of *M. tuberculosis* at concentrations of a similar order to those at which it exerts antibiotic effects.

Acknowledgments

One of us (E. Stachiewicz) is greatly indebted to McGill University for the award of a Harrison-Watson Fellowship and also to the Royal Edward Laurentian Hospital for financial assistance. We gratefully acknowledge able assistance in this work by Mr. M. Islam.

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STUDIES ON THE DISTRIBUTION AND KINETICS OF THE ALKALINE PHOSPHATASE OF RAT SMALL INTESTINE[1]

EUGENIE TRIANTAPHYLLOPOULOS AND JULES TUBA

Abstract

Alkaline phosphatase activity was demonstrated throughout the entire length of the small intestine of the albino rat and the relative amounts present seemed to decrease exponentially from the pylorus to the ileocolic valve. All subcellular fractions of intestinal homogenates were found to hydrolyze sodium β-glycerophosphate. The distribution of activity of the enzyme was as follows: "microsomes", about 76%; nuclei, 10%; mitochondria, 8%; and in the nongranular, supernatant fraction, 9%. The specific activity for these fractions was: 52, 3, 10, and 3, respectively. The effects of the time of incubation, pH of the hydrolytic mixture, and the concentration of the enzyme were similar in all fractions. For the enzyme in the various fractions slight differences were observed in the values of the Michaelis constants, and in the degree of activation by magnesium ion. These variations may be explained on the basis of differences in the accessibility of substrate and magnesium ion to the enzymes in the various fractions. The finding that the "microsomes" contained the highest levels of alkaline phosphatase suggests that this intestinal enzyme is produced almost entirely by these submicroscopic particles.

Introduction

It has been suggested that the digestion products of fats (1–5), carbohydrates (6–15), and proteins (3, 16–24) are absorbed through the walls of the small intestine of the rat by means other than simple diffusion. It is thought that, in many cases, this involves phosphorylation and dephosphorylation and that alkaline phosphatase participates in these processes (3–5, 9, 24–28).

Previous studies in our laboratory on the changes in alkaline phosphatase activity observed following ingestion of various foodstuffs (2, 4, 5, 9, 24) were confined to measurements of the enzyme activity of unfractionated homogenates from the first 10 centimeters of the small intestine of the rat. Histochemical studies (29–32) have provided some information about the distribution of the enzyme throughout intestinal tissue, but this is not the case in investigations which have been carried out in homogenates (3, 27).

Experiments in this paper deal with the distribution of intestinal alkaline phosphatase along the length of the tissue as well as with kinetics of the enzyme in various cellular fractions.

Materials and Methods

Adult male Wistar rats of approximately 300 g weight were used and were fed Purina fox checkers and water ad libitum. Animals were killed by decapitation. For the phosphatase distribution studies the entire small intestine was excised, cut into sections 10 cm long, and cleared of adherent tissues. Each section was placed in a small beaker, which was cooled in ice–water. The segments of intestine were washed by forcing a stream of cold

[1]Manuscript received July 13, 1958.
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alberta. This work was assisted by a grant from the National Research Council of Canada.

0.25 *M* sucrose solution from a wash bottle through the lumen until the washing fluid was no longer stained with bile or contaminated with intestinal contents. The thick material from the lumen of the last sections was removed with a glass rod before the tissue was washed with cold isotonic sucrose solution. The sections were then carefully blotted with a filter paper, weighed to the nearest 0.01 g, and finally homogenized in a Potter–Elvehjem homogenizer with cold 0.25 *M* sucrose solution to a final volume of 25 ml.

Preliminary fractionations of homogenates obtained from the first two sections (i.e. those nearest the pylorus) were performed in an International refrigerated centrifuge (model PR-1), according to the method of Schneider (33). This was slightly modified in order to obtain (*a*) a nuclear fraction less contaminated by mitochondria and (*b*) a more complete sedimentation of the microsomes; i.e. the nuclei were centrifuged at 600 × *g* for 5 minutes, and microsomes at 16,500 × *g* for 90 minutes. The last four fractionations were carried out in a Spinco refrigerated centrifuge (model E, Preparatory Rotor K). The combined supernatant fluids after the separation of the mitochondria were centrifuged at 63,400 × *g* for 60 minutes, and then the microsomal fraction was resuspended in cold isotonic sucrose and recentrifuged at 113,400 × *g* for 40 minutes.

Homogenates and sediments were diluted with 0.25 *M* sucrose solution to a final concentration of 10–20 units of phosphatase per liter. All operations were carried out at 2–3° C. Glassware and lusteroid tubes were always prechilled.

Alkaline phosphatase determinations were performed according to the method of Shinowara, Jones, and Reinhart (34) as modified by Gould and Schwachman (35). The unit of alkaline phosphatase activity was defined by Shinowara *et al.* as "equivalent to 1 mg of phosphorus liberated as phosphate ion during one hour of incubation at 37° C with a substrate containing sodium β-glycerophosphate, hydrolysis not exceeding 10% of the substrate, and at optimum pH 9.3 ± 0.15."

Total nitrogen was determined by the method of Hiller, Plazin, and Van Slyke (36).

Results and Discussion

(*a*) *Distribution of Alkaline Phosphatase Throughout the Small Intestine*

The points plotted in Fig. 1 represent the averaged results obtained from corresponding 10-ml sections of intestine from five rats. The variations of individual values were 5–10% of the means, except for sections 5, 6, 7, and 8 where they varied from 12–35%. It is apparent that the alkaline phosphatase activity declines exponentially with distance from the duodenum to the end of the ileum. The regression of the logarithm of the enzyme activity against distance down the intestine was calculated to be $\hat{Y} = 0.11\ X + 0.05$, i.e., the rate of decrease of the logarithm of phosphatase activity is 0.11 per section.

The deviations from the line of best fit observed in the figure are quite possibly due to differences in the total length of the five intestines used in this

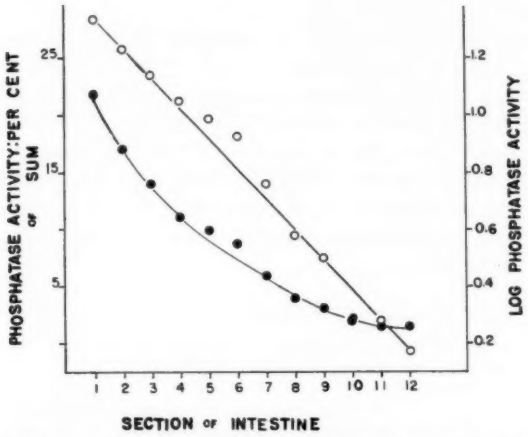

FIG. 1. Distribution of the alkaline phosphatase activity in the small intestine of the albino rat. 100% = sum of the activities of all sections. ● = % phosphatase activity. ○ = log% phosphatase activity.

experiment. The length of the small intestine varies from rat to rat. Measurements obtained from 20 animals gave a mean length of 116 ± 1.0 cm. It is evident that the classification of the intestine of the rat in 10 ml long sections does not put together strictly homologous areas from each intestine.

Another source of discrepancy is the elasticity of the tissue. The length of the small intestine changes quite easily depending on the degree of contraction of the muscular coat and the tension applied when this organ is removed from the body and straightened out for sectioning. This obviously will affect the measured length and consequently the division into sections.

The results were calculated on a weight basis. However, there is a progressive decrease in the weight of the small intestine per cm from the pylorus down. If the phosphatase values were expressed as units per cm of length, the rate of decrease would be still more marked.

It is well established (30, 37) that the villi, the absorbing units of the small intestine, are larger and more numerous in the duodenum and jejunum and that they gradually become fewer and smaller in the ileum. The pattern of alkaline phosphatase distribution throughout the small intestine considered together with the finding that the observed activity of this enzyme is increased after the ingestion of certain foodstuffs, as mentioned in the introduction, strengthens further the hypothesis of the participation of phosphatase in some absorption processes. Cytochemical studies (29–32) have shown that the striated border of the columnar epithelium is rich in alkaline phosphatase. Any biological material taken *per os* has to pass through this border in order to be absorbed; thus there is a strong possibility that it will come in contact with alkaline phosphatase during this process.

(*b*) *Fractionation Studies*

It is evident from Table I that all cellular fractions exhibit alkaline phosphatase activity. The "microsomes", the fragments of the endoplasmic reticulum, contain the bulk of the enzyme activity, 76% of the total; i.e. about 7 times the proportion contained in the nuclei, 10 times that in the mitochondria, and 8 times that in the supernatant fluid; their specific activity (phosphatase units per mg N) is 52, or about 6 times that of the total homogenate. This clearly indicates that alkaline phosphatase is definitely associated with these cellular particulates. Finally it can be noted that good recoveries are indicated in Table I.

TABLE I

Distribution of intestinal alkaline phosphatase*

	Phosphatase units/g wet intestine	Percentage of total homogenate activity	Nitrogen, mg/g of wet intestine	Specific activity: phosphatase units/mg N
(*a*) "Microsomes" sedimented at 16,500 × *g* for 90 minutes				
Nuclei	21.9± 1.4†	10.5±1.1†	7.60±0.41†	2.9±0.2†
Mitochondria	17.6± 2.2	7.5±1.0	1.60±0.12	9.6±0.9
"Microsomes"	131.5± 4.2	64.1±1.9	2.60±0.25	52.0±4.0
Supernatant fluid	35.3± 2.7	17.2±1.3	13.50±0.39	2.6±0.6
Unfractionated homogenate	214.0±10.0	100	25.20±1.30	8.4±0.6
(*b*) "Microsomes" sedimented at 63,400 × *g* for 60 minutes, washed once, and recentrifuged at 113,400 for 40 minutes				
Nuclei + mitochondria	28.0± 1.8†	15.1±1.2†		
"Microsomes"	140 ±18	76.1±9.9		
Supernatant fluid	18.4± 0.6	9.4±0.4		
Unfractionated homogenate	185.2± 5.3	100		

*Each value is the mean of the measurements for six rats.
†Standard error of the mean.

The actual alkaline phosphatase activity of the microsomal fraction within the cell may be greater than 76% of the total. In the experiments performed with the Spinco ultracentrifuge each tube had to be filled completely with solution in order to be able to withstand the high gravitational forces. As a consequence the microsomes were suspended in relatively large volumes of sucrose solution (35 ml) instead of the usual 3 ml. We found that in such cases 10–15% of the microsomal phosphatase activity, representing 8–10% of that of the total homogenate, was washed out with the sucrose solution. If this loss were taken into consideration, the microsomal phosphatase activity would be 85%. De Duve and associates (38) found that 97% of intestinal alkaline phosphatase activity in the rabbit and 83% in the guinea pig were associated with the "microsomes". These submicroscopic particles are now considered to be the main site of protein synthesis within the cell. It has been suggested that amino acids are activated by adenosine triphosphate before being incorporated into microsomal protein (39, 40, 41). The association of alkaline phosphatase with these particulate components may indicate a participation by this enzyme in some step of protein formation.

(*c*) *Kinetics*

Determination of the Reaction Order

The results are represented by Fig. 2. It is evident that the reaction is one of zero order for the first 60 minutes for the unfractionated homogenate as

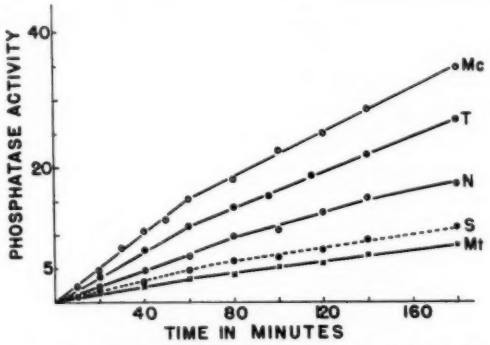

FIG. 2. Time course of intestinal alkaline phosphatase activity (γ P per ml of homogenate). Mc = microsomes, T = total homogenate, N = nuclei, S = supernatant fluid, Mt = mitochondria.

well as for the various fractions of intestinal tissue. The reaction constants obtained under the conditions described above are:

Nuclei	11.8×10^{-2} γ phosphorus/minute
Mitochondria	5.8×10^{-2} γ phosphorus/minute
Microsomes	24.8×10^{-2} γ phosphorus/minute
Supernatant	7.7×10^{-2} γ phosphorus/minute
Unfractionated homogenate	19.4×10^{-2} γ phosphorus/minute

Optimum pH

The results are plotted in Fig. 3. The effect of the pH of the incubation mixture on the activity of the intestinal alkaline phosphatase of the rat is the same for all cellular fractions with the optimum pH at 9.3 (initial substrate concentration = 0.0106 *M*).

Small amounts of *acid* phosphatase appear to be present in all cellular fractions. Determinations of this enzyme at pH 4.9 and with lower dilutions of homogenates (32–625 times) gave the results represented by Table II. The microsomal fraction again seems to exhibit the highest percentage of the total phosphatase activity, as noted with the alkaline enzyme. Berthet and de Duve (42) found that 56% of the acid phosphatase of liver homogenates is associated with mitochondria but in fresh preparations the enzyme is largely inactive and is activated after aging or after repeated freezing and thawing.

Michaelis Constant (K_m)

The effect of varying the substrate concentration is illustrated in Fig. 4*a* and Fig. 4*b*. The K_m values of alkaline phosphatase in the different fractions calculated from the experimental data for β-glycerophosphate are:

Unfractionated homogenate	0.0041 *M*
Nuclei	0.0034 *M*
Mitochondria	0.0030 *M*
Microsomes	0.0025 *M*
Supernatant fluid	0.0023 *M*

The slight differences in the values of K_m are not sufficient to indicate the presence of a different alkaline phosphatase in each fraction. If this were the case, the K_m value for the unfractionated homogenate ought to be the weighted mean of the Michaelis constants obtained from all the fractions, a value very close to the K_m of the microsomal fraction as this was shown above to contain

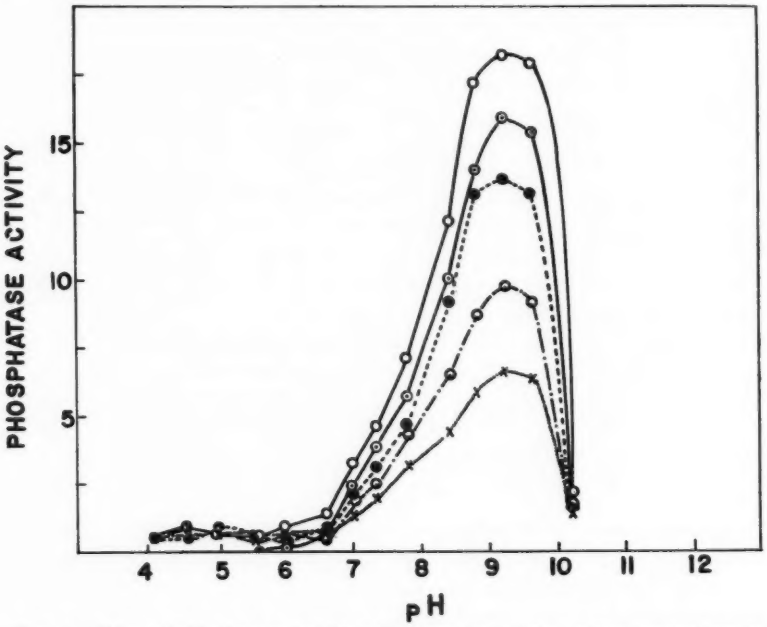

FIG. 3. Effect of pH of the hydrolysis mixture on the activity of the intestinal alkaline phosphatase of the rat (γ P per hour per ml of homogenate). ○ = total homogenate, ⊙ = microsomes, ● = supernatant fluid, ◒ = mitochondria, × = nuclei.

TABLE II

Distribution of acid phosphatase activity in the small intestine of the albino rat*

Subcellular fraction	Phosphatase units/g wet intestine	Percentage of total homogenate activity	$\frac{\text{Acid‡} \times 100}{\text{Alkaline‡}}$
Nuclei	0.24 ± 0.02†	11 ± 1†	1.1 ± 0.1†
Mitochondria	0.39 ± 0.06	18 ± 3	2.5 ± 0.6
"Microsomes"	0.86 ± 0.09	40 ± 1	0.63 ± 0.1
Supernatant fluid	0.70 ± 0.01	33 ± 1	1.2 ± 0.2
Unfractionated homogenate	2.12 ± 0.06	100	0.96 ± 0.1

*Each value is the mean of results obtained from five rats.
†Standard error of the mean.
NOTE: Acid‡ = acid phosphatase activity. Alkaline‡ = alkaline phosphatase activity.

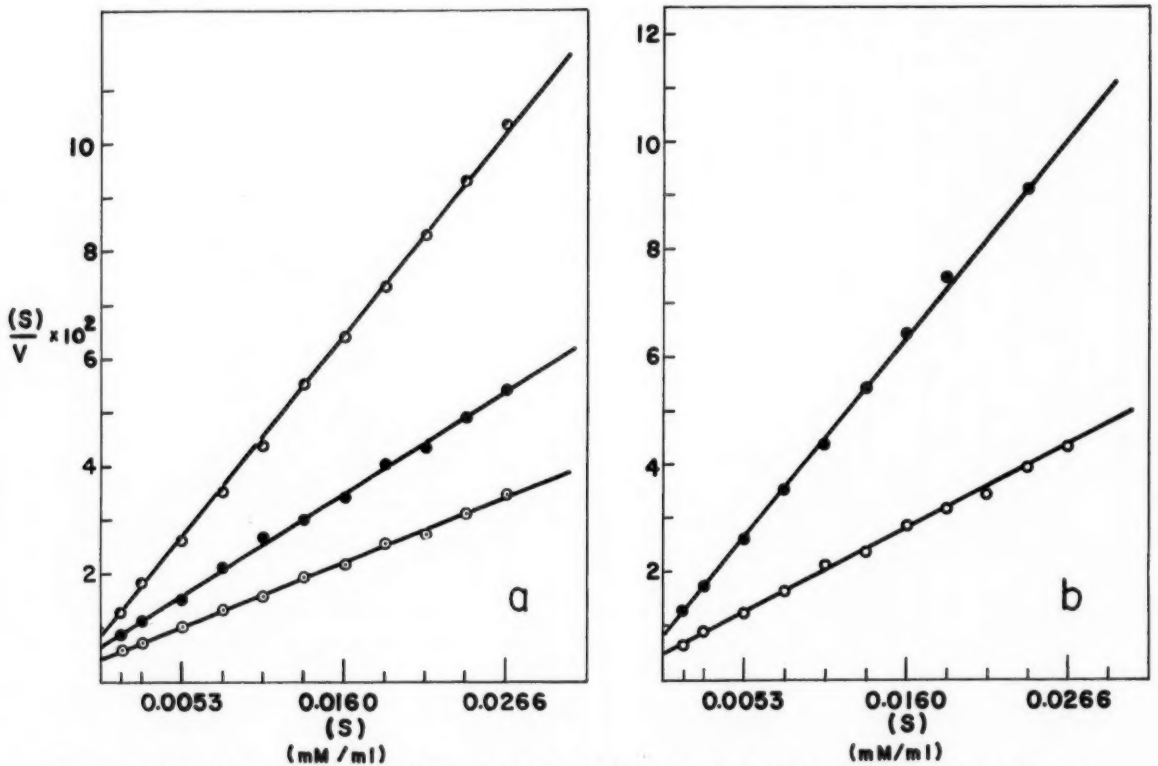

FIG. 4. Effect of substrate concentration on the activity of the intestinal alkaline phosphatase of the albino rat. (4*a*) ◒ = microsomes, ● = nuclei, ⊙ = total homogenate. (4*b*) ● = supernatant, ○ = mitochondria.

76% of the total alkaline phosphatase. The good recoveries reported in Section (*b*) of this paper rule out the possibility of any inhibition or activation of the enzyme contained in any one fraction by factors contained in another fraction.

It was noticed that the degree of homogenization had some influence on the value of the K_m of the total homogenate. Michaelis constants, determined on crude homogenates, were found to be slightly higher than for the same homogenates after they had been subjected to further treatment in the glass homogenizer. It seems probable that the slight differences in the K_m values may be due to a difference in the availability of the substrate for the enzyme in the preparations above.

In the case of the nuclei, as well as the mitochondria, the substrate has to pass through membranes in order to react with the enzyme and this should have a retarding effect on the rate of formation of the enzyme–substrate complex. The phosphatase in the supernatant fluid is mostly in the free form and consequently the K_m of this fraction has the lowest value.

In crude homogenates, many of the cells are intact. This means that the substrate has to cross the cellular membrane as well in some instances. This would impede the formation of the enzyme–substrate complex and hence increase the value of K_m.

Magnesium Ion Activation

The effect of magnesium ion concentration on the alkaline phosphatase activity of the various fractions of rat intestinal homogenate is shown in Fig. 5.

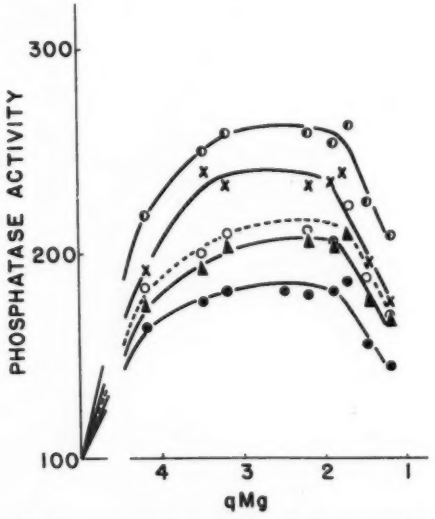

FIG. 5. Effect of Mg^{++} concentration on the alkaline phosphatase activity of subcellular fractions of intestinal homogenates. The enzyme activities are expressed as per cent of the values obtained in the absence of Mg^{++}. *q*Mg = negative logarithm of the Mg^{++} molarity in the hydrolytic mixture. ◒ = supernatant fraction, × = mitochondria, ○ = microsomes, ▲ = unfractionated homogenate, ● = nuclei.

In this figure the enzyme activity, expressed as per cent of the value obtained in the absence of Mg^{++}, is plotted against qMg. The term qMg was introduced by Jenner and Kay (43) and represents the negative logarithm of the Mg^{++} molarity in the hydrolytic mixture.

It is quite clear that Mg^{++} has a strong activating effect on the enzyme in all fractions. The increase in activity is quite pronounced even at low (Mg^{++}). The maximum activation is reached at relatively low levels of Mg^{++} and remains constant over a wide range of concentrations (3.3×10^{-4}–$2.0 \times 10^{-2}M$), after which the degree of activation becomes continuously smaller with increasing (Mg^{++}). This is in agreement with the results of Jenner and Kay (43), who studied the effect of (Mg^{++}) on dialyzed preparations of phosphatase from various tissues. Figure 5 shows that the per cent increase in activity varies for the different fractions as well as for the unfractionated intestinal homogenate. The nuclear alkaline phosphatase shows the least activation, while the enzyme in the supernatant fluid exhibits the greatest response to Mg^{++}. This may be due to differences in permeability of cellular

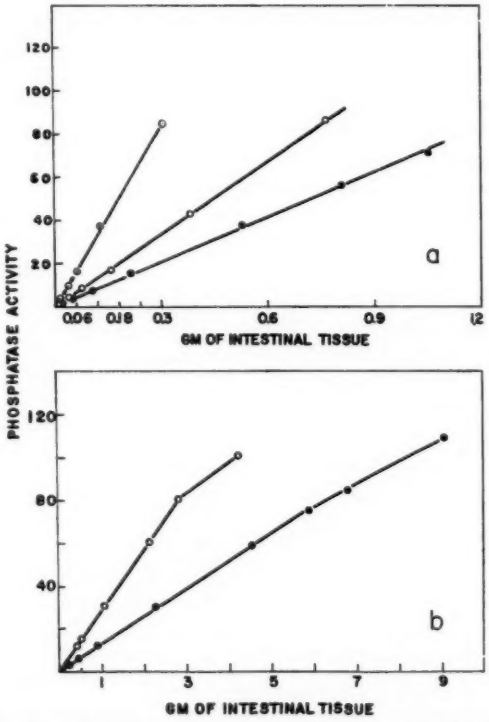

FIG. 6. Effect of enzyme concentration on the reaction rate of the intestinal alkaline phosphatase of the albino rat (γ P per hour per ml of homogenate).
(6*a*) ⊙ = total homogenate, ○ = microsomes, ● = supernatant fluid.
(6*b*) ○ = nuclei, ● = mitochondria.

and particulate membranes to Mg^{++}. In addition, the presence of other ions or factors in the various particulate components may modify the effect of Mg^{++} on alkaline phosphatase. However, the pattern of activation is much the same in all cases.

Enzyme Concentration

The effect of the enzyme concentration on the reaction rate is represented by Fig. 6*a* and Fig. 6*b*. As long as the concentration of the enzyme does not exceed 80 units per liter of homogenate a straight line is obtained by plotting activity against enzyme concentration (i.e. grams of intestinal tissue). With higher concentrations, the reaction rate is less than that expected from direct proportionality. The reasons may be (*a*) the amount of substrate is not adequate to saturate all the enzyme active centers; (*b*) hydrolysis exceeds 10% of the substrate and the accumulation of the hydrolytic products may favor the reverse reaction; (*c*) the possibility of the presence of inhibiting factors in the intestine. Such inhibitors may not be effective at low concentrations, but may retard enzyme action when they are present in large amounts.

The similarity of the kinetics of the reaction of alkaline phosphatase manifested by all subcellular fractions suggests that the same enzyme is responsible for the hydrolysis of β-glycerophosphate in all cases. This enzyme was shown to hydrolyze glucose-6-phosphate and lecithin as well (**44**). The finding that the "microsomes" contain the highest percentage of the enzyme activity indicates that alkaline phosphatase is mainly, or may be entirely, produced in the endoplasmic reticulum; it is not improbable that the enzyme detected in the other fractions originated also in the ergastoplasm.

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CHANGES IN INTESTINAL AND SERUM ALKALINE PHOSPHATASE LEVELS DURING ABSORPTION OF CERTAIN AMINO ACIDS[1]

EUGENIE TRIANTAPHYLLOPOULOS AND JULES TUBA

Abstract

After force-feeding fasted male albino rats with solutions of amino acids it was observed that:

(*a*) Intestinal alkaline phosphatase levels were significantly increased after ingestion of glycine, leucine, threonine, serine, glutamic acid, methionine, arginine, and large doses of glycine – glutamic acid – histidine mixtures, while a decrease was noted after force-feeding tryptophan.

(*b*) Serum alkaline phosphatase activity was stimulated by solutions of glycine, histidine, and glutamic acid when each amino acid was given singly and even more when a mixture of these three amino acids was force-fed. Methionine on the other hand produced a highly significant depression of the activity of this enzyme.

(*c*) Serum inorganic phosphorus levels were significantly decreased after ingestion of glycine, leucine, serine, threonine, methionine, glutamic acid, histidine, phenylalanine, tyrosine, and tryptophan.

(*d*) Serum α-amino nitrogen concentration was increased in all cases except with cystine and lysine, when no change was observed, whereas arginine produced a highly significant drop, attributed to stimulation of urea cycle.

(*e*) The α-amino nitrogen concentration of intestinal homogenates was increased after force-feeding glycine, leucine, cystine, methionine, glutamic acid, lysine, histidine, phenylalanine, and large amounts of glycine – histidine – glutamic acid mixtures.

Introduction

Ingestion of casein has been found to increase both serum (1, 2) and intestinal (2, 3, 4) alkaline phosphatase levels. Vitellin has also been reported to stimulate intestinal phosphatase activity (4). These phosphoproteins are not attacked by purified intestinal phosphatase, but inorganic phosphate (P_i) is readily liberated after preliminary treatment with crude trypsin (5). The stimulation of alkaline phosphatases could be attributed to the presence of the prosthetic groups of these two proteins. Considerable evidence from the literature, however, indicates that individual amino acids of the proteins may be responsible for this phenomenon.

Numerous experiments both in vivo (6–11) and in vitro (12–17) have shown that the L-forms of most amino acids are actively absorbed by the intestine of various animals (rats, dogs, guinea pigs, and hamsters). This active absorption of amino acids has been reported to be stimulated by the presence of P_i and adenosine triphosphate (ATP) (18, 19), while it is inhibited by anaerobiosis (14), dinitrophenol (13, 14, 19), deoxypyridoxine (14), cyanide (13), and tourniquet shock (17). It seems not improbable, therefore, that oxidative phosphorylation is involved in amino acid absorption. The increase in intestinal alkaline phosphatase levels observed after ingestion of casein and vitellin may be related to the function of the enzyme in connection with the phosphorylation of individual amino acids during absorption rather than to

[1]Manuscript received July 13, 1958.
Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alberta. This work was supported by the National Research Council of Canada.

stimulation of formation of the enzyme by the prosthetic group of these phosphoproteins. If this is the case, ingestion of single amino acids should stimulate intestinal phosphatase levels as well. The experiments to be described in this paper were designed to investigate this problem.

Materials and Methods

Adult male albino rats of the Wistar strain, which weighed 260–350 g, were used in all experiments. They were housed in individual wide-meshed cages in order to prevent coprophagy and were fasted for 3 days prior to each experiment. Water ad libitum was available. The method of force-feeding these fasted animals was adapted after considerable experimentation. It was noted that the rats became excited and nervous during the first force-feedings. It has been shown that adrenocorticotrophic (20) and adrenocortical (21, 22) hormones, the secretion of which is stimulated during stress, affect serum and intestinal alkaline phosphatases. Sodium chloride fed once and twice to rats fasted for the same length of time as the rats used in these experiments seemed to depress the levels of the intestinal phosphatase activity, while it did not produce any significant effect after three or four such feedings (Table I).

TABLE I

Effect of repeated force-feedings of 0.76 *M* solutions of NaCl on the intestinal alkaline phosphatase levels of the albino rat

Group	Intestinal alkaline phosphatase units/g wet tissue	Value of *P*
Controls	226 ± 34 (6)*	
Rats force-fed NaCl once	175 ± 22 (7)	<0.02
Rats force-fed NaCl twice	183 ± 37 (8)	<0.05
Rats force-fed NaCl 3 times	212 ± 48 (7)	>0.6
Rats force-fed NaCl 4 times	216 ± 18 (6)	>0.5

*No. of rats/group.

Thus the first two force-feedings were considered necessary to accustom the rat to the procedure and consequently minimize the possibility of stress. On the following day the third force-feeding did not produce any clear cut effect on the levels of the enzyme. If larger volumes were given at the time of the third feeding, the animals seemed uncomfortable. Preliminary experiments showed that from 2 to 4 hours, the exact time depending on each particular amino acid, were required for the complete disappearance of the force-fed solution from the stomach. It was decided, therefore, to give two separate doses of each solution at 2–4 hour intervals. In this way there was a quantity of amino acid continuously available for absorption from the time of the third force-feeding until the death of the animal. The rats appeared to be uncomfortable and developed diarrhea after they had received lysine, arginine, or tryptophan.

An ordinary 5-ml syringe with a narrow catheter, 1.5 mm in diameter, attached to it was used for the force-feedings. In the case of an insoluble amino acid, the catheter was replaced by stomach tubing of 3-mm diameter.

In some instances, it was necessary to grind the amino acid crystals in a mortar before attempting to dissolve them, and the syringe was filled with the suspension from the wider opening. By rotating the syringe and tube, crystals were prevented from adhering to or settling on the walls of the syringe and force-feeding became easier. The pH of all solutions or suspensions was adjusted to 7.

During the morning of the fourth day the animals were force-fed 2 ml of an aqueous solution or suspension containing 0.76 moles per liter of the amino acid under investigation. For sparingly soluble amino acids such as cystine, tryptophan, and tyrosine, it was necessary to reduce the concentration to 0.57 *M*, and to compensate for this by increasing the volume to 3 ml. At about 5 p.m., the force-feeding was repeated with 3 ml of the same amino acid solution (or 4 ml of the suspension of the sparingly soluble amino acids). Two more of these larger feedings were given to the animals during the next day, 2–4 hours apart, the exact interval depending on the rate of absorption of the amino acid, which was being tested.

It was found in a preliminary experiment that mixtures of glycine, histidine, and glutamic acid were absorbed very slowly, if the suspension was brought to pH 7. A much faster absorption rate was observed if the pH was left at the original value of 3.9. This mixture was force-fed to three different groups of rats: (*a*) seven animals received four doses of the mixture in a concentrated suspension (0.76 *M* for each amino acid); (*b*) six rats were given the mixture for the same number of force-feedings but in a less concentrated form (0.25 *M* for each acid); and finally (*c*), six rats received three doses of the same suspension as group (*a*). The first and the third groups which received the glycine – glutamic acid – histidine mixture were killed 3–4 hours after the fourth force-feeding.

All other animals were killed by decapitation 2 hours after the last force-feeding. The blood which flowed from the neck was collected and the serum was separated within 2 hours for the amino acid and alkaline phosphatase determinations. The first 10-cm section of the small intestine (measured from the pylorus) was carefully excised, washed out with a stream of cold 0.25 *M* sucrose solution, blotted dry lightly, and weighed to the nearest 0.01 g. Finally, the tissue cells were ruptured by the action of the Potter–Elvehjem homogenizer with demineralized water. Two milliliters of the suspension was pipetted immediately into a test tube to serve for amino acid determinations. These samples were kept frozen until the amino acid concentrations were estimated, in order to prevent autolysis, which is fairly rapid in intestinal tissue homogenates even if these are maintained at the temperature of the cold room (4° C).

Alkaline phosphatase activity in blood sera and unfractionated intestinal homogenates was determined by the micromethod of Shinowara, Jones, and Reinhart (23), as modified by Gould and Schwachman (24). The unit of phosphatase was defined (23) as equivalent to 1 mg of phosphorus liberated from Na-β-glycerophosphate during 1 hour of incubation at 37° C and pH

9.3 ± 0.15, hydrolysis not exceeding 10% of the substrate. The estimations of α-amino-nitrogen were carried out by the method of Frame, Russell, and Wilhelmi (25) as modified by Russell (26).

All amino acids used in these experiments were obtained from Nutritional Biochemicals Corporation and only the L-forms were fed.

Results

Control rats were given water or 0.76 *M* sodium chloride solution four times in the same manner as the experimental animal or were left undisturbed. It was noted in Table I above that after three or four force-feedings of sodium chloride no significant change was produced in the intestinal alkaline phosphatase activity. The control values for the results in Table II represent the results obtained from animals force-fed water or which had been left undisturbed, as there was no statistically significant difference between the two. The statistical analyses of the results presented in Table II are given in Table III.

(*a*) *Effect of Repeated Force-Feeding of Various* L-*Amino Acids on Serum and Intestinal Alkaline Phosphatases and Serum Inorganic Phosphorus* (P_i)

It is evident from Tables II and III that all of the monoamino-monocarboxylic acids that were force-fed produced a highly significant rise in rat intestinal alkaline phosphatase, and a highly significant decrease in serum P_i values. Among these only glycine increased the activity of the serum enzyme as well.

As far as the sulphur-containing amino acids are concerned, L-cystine was without any significant effect on either the alkaline phosphatase or serum P_i levels. However, L-methionine produced a highly significant elevation of intestinal phosphatase and a highly significant decrease in serum phosphatase and serum P_i.

L-Glutamic acid, like glycine, increased the values obtained for both serum and intestinal alkaline phosphatase, and decreased serum P_i.

Of the basic amino acids, L-lysine-hydrochloride was without effect throughout. L-Arginine increased the intestinal phosphatase without affecting the serum enzyme or serum P_i. L-Histidine elevated serum phosphatase and decreased serum P_i values.

Both tyrosine and phenylalanine decreased serum P_i levels, but had no effect on serum or intestinal alkaline phosphatases.

L-Tryptophan decreased significantly the activity of the intestinal alkaline phosphatase and the concentration of serum P_i.

When the glycine – L-glutamic acid – L-histidine suspension was force-fed twice on the last day (i.e. 4 times in all), and in a high concentration (0.76 *M* for each amino acid), it produced a highly significant elevation of both enzymes, but only the serum enzyme values were affected when the concentration was reduced to 0.25 *M* for each amino acid or when the total number of force-feedings was three (i.e. only one feeding during the last day of the experiment). Serum P_i was unaffected in all three groups.

TABLE II

Effect of amino acid ingestion on the levels of serum and intestinal alkaline phosphatase, serum inorganic phosphorus, and serum and intestinal amino acids

	Initial body weight (g)	Phosphatase units per:		P_i, mg/100 ml serum†	α-NH_2-N mg per:	
		g wet intestine	100 ml serum		100 g wet intestine	100 ml serum
Controls	316±3*	232±6 (38)	27.3±1.2 (30)	7.6±0.2	107.9±2.5 (34)	7.9±0.2 (28)
Glycine	308±5	292±9 (10)	34.7±2.3 (10)	6.4±0.2	124.9±5.0 (14)	9.2±0.1 (6)
L-Leucine	315±4	324±14 (14)	27.8±3.4 (14)	6.2±0.3	132±10 (14)	10.0±0.7 (6)
L-Serine	314±3	290±8 (14)	31.7±2.3 (10)	5.8±0.2	122.8±7.3 (14)	9.2±0.4 (6)
L-Threonine	311±4	270±10 (17)	29.9±2.2 (10)	5.7±0.3	119.8±5.4 (16)	18.4±1.2 (10)
L-Cystine	314±3	249±12 (14)	24.2±3.3 (10)	7.4±0.2	126.6±4.2 (13)	8.4±0.3 (9)
L-Methionine	317±5	297±8 (14)	15.9±1.6 (10)	4.9±0.2	129.3±8.6 (14)	23.5±0.3 (6)
L-Glutamic acid	314±5	291±11 (13)	37.1±1.7 (10)	5.9±0.3	132.8±5.1 (13)	11.2±0.5 (6)
L-Lysine	317±3	229±9 (12)	25.7±3.6 (4)	7.1±0.6	121.4±5.3 (12)	8.8±0.4 (4)
L-Arginine	316±4	301±14 (13)	26.8±2.5 (6)	7.7±0.3	122.8±7.0 (13)	6.5±0.2 (6)
L-Histidine	316±4	215±11 (14)	34.0±3.5 (10)	6.1±0.2	139.1±5.5 (13)	20.5±2.4 (6)
L-Phenylalanine	314±5	244±10 (16)	24.8±2.5 (8)	5.7±0.04	140.4±5.6 (14)	16.7±1.3 (8)
L-Tyrosine	312±7	223±7 (14)	27.3±3.1 (8)	6.2±0.2	120.2±6.4 (12)	9.9±0.4 (8)
L-Tryptophan	318±4	201±4 (10)	30.2±3.3 (10)	5.1±0.3	104.1±6.7 (10)	9.1±0.9 (6)
Glycine – histidine – glutamic acid (0.76 *M* each), fed 4 times		315±16 (7)	47.6±8.2 (7)	6.4±0.6	141.5±5.6 (6)	29.5±4.9 (6)
Gly – His – Glu acid (0.25 *M* each) fed 4 times		232±13 (6)	41.4±1.2 (6)	7.6±0.2	109.9±4.4 (6)	17.1±0.8 (6)
Gly – His – Glu acid (0.76 *M* each), fed 3 times		242±13 (6)	34.3±2.6 (6)	7.2±0.2	119.2±5.6 (6)	13.2±2.1 (5)

*Standard error of mean.
†Number of rats in each group of this column is same as in preceding one. Values in parentheses refer to number of rats/group.

(*b*) *Effect of Repeated Force-Feedings of Various* L-*Amino Acids on the* α-*Amino Nitrogen Concentration* (α-NH_2-N) *in Intestinal Tissue and Blood Serum*

The concentration of α-NH_2-N in the intestinal wall was significantly increased after force-feeding the following: glycine, L-leucine, L-cystine, L-methionine, L-glutamic acid, L-lysine, L-histidine, and L-phenylalanine. The glycine – L-glutamic – L-histidine mixture produced a highly significant rise in the intestinal α-NH_2-N concentration only when it was force-fed four

times and in high concentration (group (*a*)) (Tables II and III). This rise should not be considered necessarily as increased concentration of amino acids within the intestinal cells themselves. It is possible, especially in the case of insoluble and adherent amino acids, that some particles were stuck between the villi and could not be washed out during the preparation of the intestinal section.

TABLE III

Statistical analysis of data in Table I (Values of *P*)

	Phosphatase		Serum P_i	α-NH_2-N	
	Intestine	Serum		Intestine	Serum
Glycine	<0.01	<0.01	<0.01	<0.01	<0.01
L-Leucine	<0.01	>0.50	<0.01	<0.05	<0.01
L-Serine	<0.01	>0.05	<0.01	>0.05	<0.02
L-Threonine	<0.01	>0.30	<0.01	>0.05	<0.01
L-Cystine	>0.20	>0.30	>0.30	<0.01	>0.20
L-Methionine	<0.01	<0.01	<0.01	<0.05	<0.01
L-Glutamic acid	<0.01	<0.01	<0.01	<0.01	<0.01
L-Lysine	>0.50	>0.50	>0.40	<0.05	<0.05
L-Arginine	<0.01	>0.50	>0.10	>0.05	<0.01
L-Histidine	>0.10	<0.01	<0.01	<0.01	<0.01
L-Phenylalanine	>0.20	>0.30	<0.01	<0.01	<0.01
L-Tyrosine	>0.30		<0.01	>0.05	<0.01
L-Tryptophan	<0.01	>0.40	<0.01	>0.50	>0.20
Glycine – L-histidine – L-glutamic acid (0.76 *M* each), fed 4 times	<0.01	<0.01	>0.05	<0.01	<0.01
Gly – His – Glu acid (0.25 *M* each), fed 4 times	>0.50	<0.01	>0.10	>0.50	<0.01
Gly – His – Glu acid (0.76 *M* each), fed 3 times	>0.50	<0.02	>0.10	>0.05	<0.03

In the blood serum no change in the values of α-NH_2-N followed the force-feeding of L-cystine and L-tryptophan. All the other amino acids produced significant increases in the serum concentration of α-NH_2-N, except L-arginine which produced a highly significant decrease.

Considerable amounts of the N reported here as serum α-NH_2-N must have been actually ammonia-N, as ammonia is freely formed during the deamination of amino acids and its N also reacts with the naphthoquinone, the reagent of Frame *et al.* (24). The reliability of the results as a measure of amino acid absorption and accumulation in the blood, however, does not suffer as the ammonia-N in this case originates in the force-fed amino acid. The amount of ammonia in the serum and intestinal homogenates of the normal and fasted animals is negligible.

Discussion

As pointed out in the introduction there is considerable indication in the literature that phosphorylation takes place during the absorption of certain L-amino acids. The increases in intestinal phosphatase levels, which were observed in our experiments after the force-feeding of certain amino acids and which were accompanied by a decrease in serum P_i values (except in the case of arginine), provide further support for this hypothesis. Zamecnik and coworkers (27, 28) have advanced the idea of carboxyl activation of amino acids during incorporation into the microsomal proteins. A similar phenomenon may well take place during intestinal absorption. Under aerobic conditions and in the presence of ATP, intestinal homogenates have been shown to synthesize labile phosphate derivatives of amino acids (19).

The exact role of alkaline phosphatase in amino acid absorption is not clear at the present time. Until recently this enzyme was considered almost exclusively as hydrolytic. Any increase in its activity in the body was usually interpreted as indicative of increased hydrolysis of phosphomonoesters. The synthetic ability of crude phosphatase preparations from various organs had been demonstrated by Martland and Robison (29) in 1927 and by Kay (30) in 1928, but this was later attributed to contamination by other enzymes, namely phosphokinases. A decade ago, however, Green and Meyerhof (31) showed that synthesis of phosphomonoesters from chemically pure sugar and P_i can be carried out in the presence of purified preparations of intestinal phosphatase. Evidence of transphosphorylating ability of phosphatases has also been presented by these authors (32–34), as well as by Axelrod (35, 36) and Morton (37).

Gould (38) and Madsen and Tuba (39) have shown that the marked elevation of the serum alkaline phosphatase levels after ingestion of high fat diets most likely originates in the intestine. The increased serum phosphatase activity following the force-feeding of glycine and glutamic acid in our experiments may also be of intestinal origin. It is not unlikely that translocation of the intestinal enzyme occurred in the case of the other amino acids which elevated the values of intestinal phosphatase, but at a time not included in this investigation.

The depressing effect of methionine on serum alkaline phosphatase was first observed in this laboratory a decade ago (40, 41). Choline also had been reported (42, 43) to produce a similar change. This effect seems to be connected with the lipotropic properties of these substances.

It has been shown that amino acids may act as activators or inhibitors of alkaline phosphatases (44–46). Hence the suggestion could be made that the elevation of phosphatase levels observed in our experiments was not really due to an increased enzyme production by the animal (adaptation to increased demands), but to an in vitro activation of phosphatase by the high amino acid concentration during the phosphatase determinations. The levels of α-NH_2-N in the intestinal homogenates during incubation were all between 2.0 and 2.8×10^{-6} M, i.e. less than 2% of the concentrations required for

activation and much less than those required for inhibition. The serum concentration of α-NH_2-N in the experiments with threonine, methionine, histidine, phenylalanine, and the glycine – L-histidine – L-glutamic acid mixture were in the range where activation should be expected, if the serum phosphatase shows the same sensitivity towards amino acids as intestinal, bone, and kidney phosphatases do. However, only histidine and the mixture of the three amino acids increased serum phosphatase activity significantly. No significant effect was exerted by phenylalanine or threonine, while a decrease was produced by methionine. A possibility which we cannot exclude, although there is no evidence for it at present, is that sufficient amounts of amino acids were, in some cases, already bound to the enzyme and thus could not be determined as free α-NH_2-N, but could exert their activating or inhibiting action on it.

The highly significant decrease in serum α-NH_2-N concentration observed after force-feeding of arginine is undoubtedly due to an abundant formation of ornithine from arginine and stimulation of the urea cycle. This action of arginine has been reported (47, 48) to be the reason for the beneficial effect which this amino acid exerted during toxicity experiments with lethal doses of mixtures of other amino acids.

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NOTES

NOTE ON A POSSIBLE SOURCE OF ERROR IN COUNTING RADIOACTIVE SAMPLES[1]

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With the technical assistance of A. LÉVEILLÉ

In the course of an experiment in which the incorporation of C^{14} from acetate-1-C^{14} into the cholesterol of different tissues was being studied, it was noted that some digitonide samples, of known low total activity (e.g. aorta cholesterol) were losing part of this "activity" when the counting was repeated; for instance, a sample that gave 80 c.p.m. immediately after being plated was down to 24 c.p.m. 4 hours later. The samples were collected on disks of filter paper (2) by the filtration apparatus of Henriques *et al.* (1) and the filter paper used was 589 Blue Ribbon (Schleicher & Shuell).

Analysis of the Phenomenon

A systematic verification of the methods and manipulations involved in preparing the cholesterol digitonide samples was undertaken.

First, tissues from animals not injected with 1-C^{14}-acetate were carried through all the different steps of cholesterol extraction: reflux for 5 hours in alcoholic KOH, extraction with petroleum ether, drying with sodium sulphate for 5 hours, evaporation to dryness under reduced pressure in nitrogen atmosphere, dissolution in alcohol–acetone, precipitation with digitonin, plating on filter paper planchets in a suction apparatus, drying under infrared lamps. The samples so prepared were immediately placed in a gas-flow end-window counter (Nuclear Chicago, Model D-47) and were found to be active, much in excess of the background; here again, the activity decreased with time. Such a procedure had shown, then, that the phenomenon could be duplicated without any radioactive substance to start with.

Next, the extraction procedure (again on tissues of animals not injected with radioactive material) was repeated but carried only as far as the precipitation stage: the precipitates, merely filtered by gravity, did not show, when placed in the counter, any of the "activity" as described above. This eliminated all the previous stages: refluxing, extraction, drying with sodium sulphate, evaporation, and the precipitation by digitonin itself, as being the cause of the observed phenomenon.

The investigation was then focussed on the group of operations called "Plating". A step-by-step analysis eliminated the washing solutions (alcohol,

[1]With financial assistance from the Defence Research Board of Canada (D.R.B. grant 9310-06).

[2]Medical Fellow from the National Research Council of Canada.

acetone) and the infrared drying lamp. Consequently, the problem became limited to the suction apparatus itself (1); a completely new system was used and placed in a room where radioactive material had never been used.

It was found that the filter paper itself without any other substance or treatment, except the one of being submitted to suction, gave a number of c.p.m. much above that of the background when placed in the counter immediately after suction. The phenomenon could be repeated at will, provided that the filter paper was submitted to suction. It was also found that the longer the suction, the greater was the initial "activity" of the blanks. Figure 1 shows typical "decay" curves obtained for the activity on filter papers submitted respectively to 80 and 10 minutes of suction.

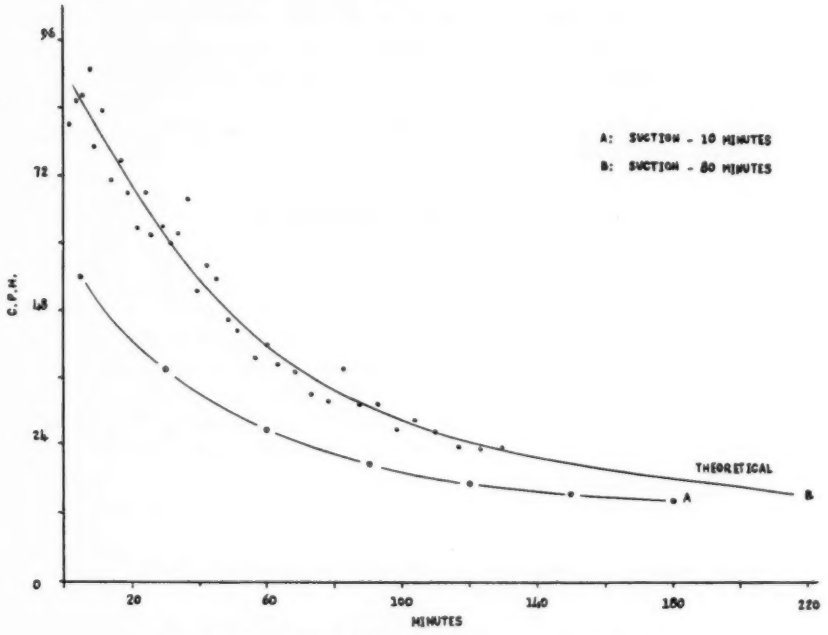

FIG. 1. Recorded activity curve (background: 13 c.p.m.).

Discussion

Dust filtered from the atmosphere contains natural radioactivity due to the disintegration products of radon and thoron; the decay curve of these disintegration products has been measured at the X-Rays and Nuclear Radiations Laboratory of the National Research Council of Canada (3). Such a decay curve and the one that we have obtained for the activity on a filter paper submitted to 80 minutes of suction (Fig. 1) have been compared, and the two curves were found to be almost identical in shape for the first 60 minutes and to differ only slightly over the next 60 minutes (3).

Conclusion

It would appear, therefore, that the effect we observed is due to the natural radioactivity present on atmospheric dust.

We thought that it was of interest to report this possible source of error to other workers in the field using the same method. Obviously, the only necessary precaution is to wait at least four hours before counting a plated sample, especially if the latter is of low activity.

Acknowledgment

We are very grateful to Dr. G. N. Whyte of the X-Rays and Nuclear Radiations Laboratory of the National Research Council for studying the decay curves described in this note and bringing to our attention the similarity between them and the ones that he has measured for the disintegration products of radon and thoron found on dust filtered from the atmosphere.

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Received June 27, 1958.
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Canadian Journal of Biochemistry and Physiology

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